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MORPHOLOGY, BUOYANT DENSITY AND POLYPEPTIDE COMPOSITION OF TEN ISOLATES OF INFECTIOUS PANCREATIC NECROSIS VIRUS

BY

© NICHOLAS NYUK-FAH CHANG

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Morphology, Buoyant Density and Polypeptide Composition of Ten Isolates of Infectious Pancreatic Necrosis Virus submitted by Nicholas Nyuk-Fah Chang in partial fulfilment of the requirements for the degree of Master of Science in Microbiology.

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ABSTRACT

Ten isolates of infectious pancreatic necrosis (IPN) virus were compared with respect to their morphology, buoyant density in CsCl and polypeptide composition. Initial studies were undertaken to determine firstly the reliability and efficiency of employing chinook salmon embryo (CHSE-214) cells as a cell culturs system for the study of IPN virus and secondly, to establish reliable methods for the concentration and purification of the virus for physico-chemical analysis.

Comparative studies on the virus dose-response curves of IPN virus in CHSE-214 cells and rainbow trout (RTG-2) cells showed that CHSE-214 cells were efficient and reliable for quantitative assay of IPN virus infectivity by the plaque assay method. This cell line was also shown to be efficient in the propagation of IPN virus and for the production of virus. Under optimal conditions, infection of cells in Roux bottles yielded an average of 2.5×10^8 PFU/ml of culture medium.

One-step growth curves of VR-299 and Jasper IPN virus were followed in CHSE-214 cells. A latent period of 3 hours was detected for VR-299 and 2 hours for Jasper IPN virus. Maximum virus production was attained at 10 to 24 hours postinfection. At 24 hours after infection, 35 to 45% of the virus remained cell-associated.

Concentration and purification of IPN virus was achieved by the methods of (1) fluorocarbon extraction of virus-cell mixtures, (2) polyethylene glycol precipitation of virus in the culture medium; both methods resulted in virus concentrates that could be further purified and concentrated by two cycles of CsCl gradient centrifugation with little loss of infectivity. These purification methods have allowed the purification of

10 isolates of IPN virus and a comparison of some of their physico-chemical properties.

The morphology of all 10 IPN virus isolates in negatively stained preparations was identical when observed under the electron microscope. They were isometric and hexagonal in profile, exhibited identical particle diameters of 74 nm when compared to 88 nm latex particles and did not contain any inner capsid structure characteristic of the Reoviridae. Particle counts showed that one plaque forming unit was equivalent to 50-200 virus particles.

The buoyant densities of all the virus isolates were identical at 1.33 g/cc and their ultraviolet absorption ratios at 260/280 nm were found to be 1.20 to 1.25.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified virions of all the isolates revealed the presence of 3 polypeptides of molecular weights 50,000, 30,000, 29,000 daltons which were designated VP50, VP30 and VP29 respectively. No major differences in polypeptide composition were detected among the IPN virus isolates. VP50 constituted approximately 60% of the total protein, while the amounts of VP30 and VP29 varied slightly from isolate to isolate, but the sum of the two constituted approximately 40% of the total protein.

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Science is a very human form of knowledge. We are always at the brink of the known, we always feel for what is to be hoped. Every judgement in science stands on the edge of error and is personal. Science is a tribute to what we can know although we are fallible .....

J. Bronowski

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ABBREVIATIONS

cpe	cytopathic effect
EDTA	(Ethylenedinitrilo)tetraacetic acid, disodium salt
FCS	fetal calf serum
g	gravity (centrifuged force)
HEPES	N-2-Hydroxyethylpiperazine-N^{1}2-ethanesulfonic acid
HBSS	Hank's balanced salt solution
h	hour
IU	international units (of penicillin)
M	molar
min	minute
µg	microgram
µl	microliter
MEM	minimal essential medium
mmol	millimole
PEG	polyethylene glycol
PFU	plaque forming unit
PTA	phosphotungstic acid
pi	postinfection
PBS	phosphate salt buffer
SDS	sodium dodecyl sulfate
SSC	saline sodium citrate
Tm	melting temperature
Tris	tris(Hydroxmethyl)-Aminomethane
rev/min	revolutions per minute

INTRODUCTION

1. Infectious Pancreatic Necrosis (IPN) Disease

Infectious Pancreatic Necrosis (IPN) is an acute, highly contagious virus infection of salmonids which results in high mortality in very young hatchery-propagated trout. This acute disease was first described by M'Gonigle in 1940. His description of the disease included only the behavioural responses and gross internal lesions. The infected fish displayed a 'cockscrew' whirling about the horizontal axis, swimming eratically while descending to the bottom of the tank. This behaviour was followed by a rapid rate of respiration culminating in death. No histological examination was made in this study by M'Gonigle. A more thorough study by Wood *et al*. in 1955 revealed additional manifestations which included an increase in mucus secretion of the upper intestinal tract and the cessation of bile flow. Degeneration of the acinar and islet tissues of the pancreas was severe. Cellular detritis was widespread throughout many areas of the pancreatic tissues. Monocytes and polymorphonuclear leukocytes infiltrated into the pancreatic tissues. The largest and healthiest fingerlings became affected most acutely. The intestine was found to be free of food material, was distended and contained a clear and colourless mucus.

The infectious nature of the disease was experimentally demonstrated by Wood *et al*. in 1955 and by Sneiszko *et al*. in 1959. The latter workers showed that IPN disease could be experimentally transmitted by feeding susceptible brook trout homogenates obtained from IPN-infected trout fry. The virus was isolated in 1957 and the viral etiology of the disease was demonstrated by Wolf *et al*. (1960a; 1960b). The virus was

isolated from brook trout and was propagated in rainbow trout gonad (RTG-2) cells. To date, the virus remains unclassified primarily because of the uncertainty of the structure of its nucleic acid. Only recently have sufficient data been available to properly assign the virus to a group. The evidence indicates that it is a Diplornavirus (Cohen et al., 1973; Dobos, 1976; Macdonald and Yamamoto, 1977).

2. Fish mortality, transmission and isolation of IPN virus

In young infected salmonid fishes (fry), the mortality was reported to be high (Wolf et al., 1961). Reports on IPN disease outbreaks showed that losses can be as high as 70% among fingerling trout several weeks old (Sneiszko, et al., 1959) and up to 86% loss was reported in 8 week old trout (Salvelinus fontinalis) by Wolf et al. (1960a). In older fish, the mortality rate was reduced and symptoms of the disease were absent (Sneiszko et al., 1959). In all outward appearances infected adult fish appear normal, but shed virus with feces, eggs and sperms (Wolf et al., 1963). Thus, many of the fish that recovered became persistent carriers of the virus, the virus being shed into water with reproductive products during spawning (Wolf et al., 1961; Wolf et al., 1968; Billi and Wolf, 1969). Infection can thus be transmitted through water (Wood, Sneiszko and Yasutake, 1955), by intraperitoneal injection (Wolf, 1966) and by protozoan parasites (Moewuss-Kobb, 1965). The virus can also be transmitted from one generation to another through contaminated eggs (Wolf, Quimby and Bradford, 1963). In addition, Wolf et al. (1963) have shown that IPN virus can be transmitted in ovarian fluid from brook trout, with titres that ranged from 10^0 to $10^{6.5}$ $TCID_{50}$/ml of fluid. The majority of the virus was therefore present as an external egg contaminant. Both contact and vertical transmission are implicated in hatchery outbreaks.

Passive tranfers have been reported by Sonstegard and McDermott (1972) in both avian and mammalian species. In addition, Eskilden and Vestergaard Jørgensen (1973) isolated IPN virus from orally infected black-headed gulls (Larus redibundu) after a week's exposure to IPN virus. These reports indicate the possible transfer of IPN virus by mechanical means. Whether there is any likelihood of avian or mammalian species acting as vectors of the disease is unknown.

The diet was found to contribute to IPN disease. Brine shrimp diet that was considered to be a superior to other fish diets (e.g. beef liver) for young fry was found to significantly increase mortalities in IPN-infected fish (Sneiszko et al., 1959). Fry, when fed on this diet gained weight more rapidly than on other diets: thus, rapid growth could contribute to the increase in susceptibility to virus infections.

Various isolates of IPN virus were reported to differ in their virulence depending upon temperature (Frantsi and Savan, 1971a). The authors reported that in 2-month-old brook trout infected with an IPN virus isolate designated PEM-1, mortality was 74% at 10°C, and 46% at 15.5°C. In contrast mortality was only 31% at 15.5°C when the fish were infected with the reference IPN virus isolate VR-299 (ATCC designation), while at 10°C the mortality was only 10%. The serological relationship between PEM-1 and VR-299 IPN virus is not known.

The relationship between the presence of IPN virus in parent fish and the appearance of IPN disease in the offspring has not been thoroughly studied, and although one parent-to-offspring study was actually reported (Wolf et al., 1968), the data did not allow any conclusions to be drawn. In infected fish, IPN virus can be isolated from organs, feces, and peritoneal washings (Wolf, 1966), with a higher frequency of isolation

from feces than from peritoneal washings of brook trout (Billi and Wolf, 1969). Other studies showed that the virus was isolated more frequently from organs than from feces, particularly from the kidneys (Frantsi and Savan, 1971a; Yamamoto, 1974) in rainbow and brook trout.

3. Host range susceptibility and worldwide distribution of IPN virus

Brook trout is regarded as the principal host for IPN virus infection. Epizootics have occurred in rainbow trout (Parisot and Yasutake, 1963), brown trout (Salmo trutta, Wolf et al., 1960a), in cutthroat trout (Salmo clarkii, Parisot et al., 1963) and asymptomatic infection in atlantic salmon (Salmo salar, Mackelvie and Artsob, 1969). Although salmonids are the primary host for IPN infection, the virus has been isolated from white suckers (Catostomus commersoni, Sonstegard et al., 1972). This was probably due to a passive contamination from water which had its source from an IPN virus-infected hatachery. In experimental transfers chinook salmon (Onchorhynchus tshawytsha), kokanee (O. nerka) and coho salmon (O. kisutch) were not susceptible to the virus when inoculated (Parisot et al., 1963). IPN virus has however been isolated from an apparently healthy adult female coho salmon (McMichael et al., 1975) and from coho salmon fingerlings (Wolf and Pettijohn, 1970).

IPN virus has been isolated from virtually every trout producing area of the world. Since the first recognized outbreak of IPN disease in fingerling trout in 1963, the disease has spread widely on the North American continent. The causative virus was isolated in the United States in 1957 (Wolf et al., 1960a and 1960b) and in Canada in 1968 (Mackelvie and Artsob, 1969). The disease has been reported in Ontario (Sonstegard and McDermott, 1971) and the virus was isolated from fish stocks in Ontario (Frantsi and Savan, 1971a), in Quebec (Desautel and McKelvie, 1975)

and in Alberta (Yamamoto, 1974). A virological survey of the Canadian Maritime Provinces revealed that of 13 hatcheries examined, 9 were positive for IPN (Mackelvie and Artsob, 1969).

The first European outbreaks of IPN disease were reported in Southern France in 1964 (Wolf and Quimby, 1970, 1971; Besse and de Kinkelin, 1965; de Kinkelin and Besse, 1966) and by the following year, other epizootics were reported in other parts of the country. Diagnosis were based on gross clinical symptoms and histological examination. Since then, epizootics had occurred in Denmark (Vestergard Jørgensen and Bregnballe, 1969), in Italy (Ghittino, 1968), in Scotland (Ball et al., 1971) and in Japan (Sano, 1971). In all instances the disease was thought to be associated with infected fish or infected fish products (e.g. eggs) from North America and from known sources in Europe. In the Danish and Italian outbreaks for instance, eggs from contaminated hatcheries in France were exported to the Danish and Italian hatcheries just prior to the IPN outbreaks; while in the Scottish outbreak, the farm where the epizootics occurred had received four shipments of eggs from Denmark (Ball et al., 1971). The Japanese outbreak was due to inportation of infected rainbow trout from western United States (Sano, 1971) where IPN virus has been isolated in several of the western States (Parisot et al., 1963, 1965).

The worldwide occurrence of IPN disease and the catastrophic consequences of salmonid hatcheries has made the disease difficult if not impossible to eradicate. The best practical solution would seem to be to employ preventative measures in hatcheries that are still free from the disease. This can be achieved by obtaining eggs and sperm from IPN virus-free brood stock as well as the use of disinfection procedures in hatcheries.

4. Serological relationship between IPN virus isolates

The appearance of IPN virus in different regions of the world leads to the question of the relationship of these virus isolates. In North America, several reports have indicated that there may be serological similarities and differences between the North American isolates (Malsberger and Cerini, 1963; Wolf et al., 1969; Mackelvie and Artsob, 1969). That these isolates appear to be serologically different has been implicated only by cross-neutralization tests. Only few isolates can be said to be antigenically distinct on the basis of reciprocal cross-neutralization tests. These include the Danish isolates SP and AB (Vestergard Jørgensen and Grauballe, 1971), VR-299 and the French isolates Bonnamy, d'Honnincthun, Kerlo and Charreau (Wolf and Quimby, 1971). Wolf and Quimby reported that all the French isolates were only slightly neutralized by rabbit antiserum prepared against VR-299 IPN virus. On this basis, the authors concluded that the French isolates were only distantly related to VR-299. The serological relationship among the French isolates is more difficult to evaluate since reciprocal cross-neutralization test was performed only for the Bonnamy and d'Honnincthun isolates. They appear to be closely related to one another (Wolf and Quimby, 1971). The data on the French isolates revealed that neutralization tests were not performed with a uniform amount of virus throughout the comparative experiments. For example, using anti-Bonnamy antiserum, a 1:363 dilution of serum was found to neutralize 793 ID_{50} of Bonnamy virus/ml, while a 1:2,884 dilution of the same antiserum was required to neutralize 50 ID_{50} of Charreau virus/ml/ The results would have been more meaningful if the same quantity of virus had been used.

Attempts to serotype the various isolates of IPN virus must be reassessed in the light of recent findings by McMichael et al. (1975). These authors compared the neutralization of three IPN virus isolates (VR-299, COHO and CTT) using antiserum prepared against each isolate. VR-299 antiserum was found to neutralize the isolates in the order VR-299, COHO and CTT, i.e. VR-299 required the least amount of antibody for neutralization, while CTT required the most. However, the neutralization order was the same when COHO or CTT specific antiserum was used. These results indicated that a factor other than antigenic variation could affect the neutralization titre, and that this factor may be related to the amount of non-infectious virus present in the virus sample. Thus, the results of McMichael et al. indicated that reciprocal cross-neutralization test must be performed before any serological conclusions can be drawn. Since only a few virus isolates have been tested in this manner (Wolf and Quimby, 1971; Vestergard Jørgensen and Grauballe, 1971; Malsberger and Cerini, 1963; McMichael et al., 1975), a definite conclusion concerning antigenic variation is limited to only such isolates. A comparison of the IPN virus isolates using physio-chemical methods and purified virions may therefore be one practical approach in determining the structural relationship between the various isolates of IPN virus. The evidence to date indicates that while a number of IPN virus isolates are antigenically distinct, more careful reciprocal cross-neutralization tests with controlled amounts of input virions must be performed to determine the serological relationship of other IPN virus isolates.

5. Disinfection and inactivation of IPN virus

Despite the use of disinfectants such as iodine and chlorine, the virus has not been eliminated from infected eggs and epizootics have

occurred among offsprings of such treated eggs (Wolf and Quimby, 1971). Virus transmission may therefore be due to shipment of contaminated eggs from carrier brood fish. Contamination of eggs arises from the failure to eliminate the virus from infected hatcheries and from carrier fish. It is only recently that studies have been initiated to determine the rate of IPN virus survival in freshwater, other water systems (Tu et al., 1975; Desautel and Mackelvie, 1975; Mackelvie and Desautel, 1975) and the effect of disinfectants such as chlorine, iodine and formalin on the virus (Desautel and Mackelvie, 1975; Mackelvie and Desautel, 1975; Vestergard Jorgensen, 1973).

Tu et al. (1975) reported that VR-299 and Kamas brook trout IPN virus were stable in stream and well water for about 10 days at 4°C and 5 days at 15°C, after which time the loss in infectivity was exponential over a period of 40 days. Studies by Desautel and Mackelvie (1975) showed that VR-299 IPN virus lost 99% of its infectivity at 4°C over a period of 12 weeks in freshwater.

Disinfection studies by Desautel and Mackelvie (1975) and Mackelvie and Desautel (1975) showed that a concentration of 40 ppm of chlorine was required to inactivate $10^{7.5}$ $TCID_{50}$ of virus/ml in 30 min, while 30 ppm of active iodine (Wescodyne) was sufficient to inactivate the virus completely in 5 min. In both cases however, the concentration of virus and the contact period between the virus and the disinfectant can significantly alter the outcome of the treatment. The practical implications of these studies are as yet uncertain since the short and long term effects of disinfectants on the health of the fish have yet to be determined.

Inactivation studies by Desautel and Mackelvie (1975) and Mackelvie and Desautel (1975) showed that VR-299 IPN virus was sensitive to heat (60°C), UV radiation and formalin. More than 90% of virus infectivity was lost upon inactivation by all three methods.

6. Stability of IPN Virus

IPN virus is stable in glycerol (Wolf and Quimby, 1971), at -20 and -60°C (Malsberger and Cerini, 1963; McMichael et al., 1975) but at 4°C, stability varied depending on the isolates. For instance, of the French isolates, Bonnamy, Charreau and d'Honnincthun could be reisolated after 4 years storage in glycerol, but the Kerlo isolate showed no infectivity after 3 month storage (Wolf and Quimby, 1971).

IPN virus is stable at pH 7.0 and 7.8 when held at 5°C (Wolf and Quimby, 1971) and is resistant to pH 2.5 but labile to pH 12 (Wolf, 1976). Mackelvie and Desautel (1975) on the other hand reported that VR-299 IPN virus was sensitive to pH 2.0 and 9.0 with more than 99.9% of the infectivity lost over period of 5 weeks. The Danish isolate SP was found to be stable at pH 2.5 for 1 h with virtually no loss of infectivity (Vestergard Jørgensen, 1973). No data are available regarding the other isolates of IPN virus.

7. Propagation of IPN virus in cell culture

In the laboratory, IPN virus has been successfully propagated in primary cell cultures from rainbow trout (Salmo gaidneri); brook trout (Salvelinus fontinalis); blue gill (Lepomis machrochirus); goldfish (Carrasius auratus); and is well documented by Wolf et al. (1960b, 1964). Virus replication resulting in cpe has been described for the following established cell lines: RTG-2 from rainbow trout gonad (Malsberger and Cerini, 1963; Wolf, 1964); FHM from fathead minnow (Pimephales promelas,

Gravell and Malsberger, 1965); BF-2 from bluegill (Argot, 1969); GF from salt water blue-striped grunt fin (Hermilon scuirus, Moewuss-Kobb, 1965); SWT from swordtail, a freshwater tropical fish (Xiphophorus helleric, Kelly and Loh, 1971; Kelly, 1972); AS from the organs of the atlantic salmon (Piper, Nicholson and Dunn, 1973) and CHSE-214 cells from chinook salmon embryo (Fryer et al., 1965). The virus does not appear to replicate in mammalian cells (Wolf et al., 1960a). It was reported to cause lesions in the infected pancreas of Swiss suckling mice (Angiolelli and Rio, 1971). The authors demonstrated that both filtered and unfiltered homogenates of infected experimental mouse pancreas produced cpe in RTG-2 cells that was characteristic of IPN virus infection, while normal mouse pancreas did not produce cpe in RTG-2 cells. To date, no attempts have been made to repeat their work.

Although numerous cell lines are available for the study of IPN virus, for example the SWT (swordtail) by Kelly and Loh (1975), AS (atlantic salmon) by Piper et al. (1973), BF-2 (bluegill fry) by Argot and Malsberger (1972) and FHM (fathead minnow) cells by Scherrer and Cohen (1975), the most routinely employed cell line has been RTG-2 cells and it is considered the standard cell line for IPN virus. The virus susceptibility of RTG-2 cells is not limited to IPN virus alone. Other fish viruses have been shown to infect and cause cpe in this cell line, for example the Egtved virus (Wolf and Vestergard Jørgensen, 1970) and the IHN virus (Wolf and Quimby, 1973). The resultant cpe observed in plaques is characteristic of the virus that caused it.

The presence of more than one type of virus may suppress the plaquing efficiency of one of the viruses in a particular cell line, and consequently gives an inaccurate result when infectivity titres are

determined. This was well exemplified by a double infection study by Wolf and Vestergard Jørgensen (1970). When RTG-2 cells were doubly infected with Egtved and IPN virus, less IPN virus was adsorbed in doubly infected cultures than in singly infected cultures and the production of IPN virus was somewhat suppressed in doubly infected cultures. Interference of one virus by another is not an unusual phenomenon, but what was more important in the double infection study was that fewer plaques were formed by IPN virus in the presence of Egtved virus. The availability of other cell lines will therefore allow a more critical analysis of the data obtained. A comparison of the results from two or more different cell lines would certainly be advantageous.

IPN virus replicates at temperatures from 5 to 25°C in susceptible cells (Wolf et al., 1960a, 1960b; Malsberger and Cerini, 1963; Wolf, 1964; Gravell and Malsberger, 1965; Argot, 1969; Kelly and Loh, 1975). Infection of culture cells always results in cpe that is characterized by pycnotic cell nuclei (Wolf et al., 1960a). Cytoplasmic inclusion bodies have been reported in infected cells (Wolf et al, 1960a; Parisot et al., 1963). The inclusions appeared late in the infection period and were numerous (Parisot et al., 1965). Basophilic cytoplasmic inclusion bodies were observed in histological preparations of pancreatic acinar cells of IPN virus-infected trout (Wood, Sneiszko and Yasutake, 1955) as well as in primary trout cells infected with the virus (Wolf et al., 1960a; Parisot et al,. 1963). Cytoplasmic inclusions have been revealed in electron microscopy of thin-sectioned preparations from pancreatic tissues of naturally infected trout (Lightner and Post, 1969). Many of the structures comprised what appeared to be crystalline aggregates of the virions as well as cell debris plus virions. The relationship

of these inclusions of viral synthesis is not apparent. Whether the inclusions were viral antigens or viral coded products was not determined. Virus antigens in infected RTG-2 and AS cells have been detected by the fluorescent antibody technique (Piper et al., 1973). Initial cytoplasmic fluorescent was detected 3 to 4 h before any cpe was evident. The percentage of cells that showed fluorescence increased over the next 6 to 7 h until 80% of cells exhibited specific staining at 9 to 11 h pi. During this period, infectious virus was being produced. Fluorescence in infected cells was limited to the cytoplasm and appeared to be consistent with observations from electron microscopy (Moss and Gravell, 1969), cytochemical and autoradiographic studies (Nicholson, 1971; Argot and Malsberger, 1972) which indicated that replication of IPN virus occurred solely in the cytoplasm of infected cells.

8. Growth curve of IPN virus

The growth characteristics of IPN virus in several cell lines have been documented. These include RTG-2 (Malsberger and Cerini, 1963; Moss and Gravell, 1969); AS (Piper et al., 1973); SWT (Kelly and Loh, 1975); GF (Moewus-Kobb, 1965) and FHM (Bonnadiere et al., 1976). In RTG-2 cells, one-step growth curves determined by Malsberger and Cerini (1963) and Moss and Gravell (1969) showed that immediately following infection, there was a latent period of 5 h. This was followed by an exponential increase in intracellular virus up to 15 h pi. An increase in cell-free virus followed closely the exponential increase in intracellular virus. Maximum virus production was attained by 15 h pi, and virus titre began levelling off until termination of the experiment. The virus yield was between 200-700 $TCID_{50}$/cell.

In infected RTG-2 cells, cpe was first detected at 8 h pi (Malsberger and Cerini, 1963). The cell cytoplasm retracted, resulting in loss of the typical spindle-shaped fibroblastic morphology. Spaces or holes appeared between the cells. The author defined this stage of infection as (1+) cpe. By 10 h pi, increasing granularity of the cytoplasm was evident, and this was defined as (2+) cpe. By 12 h pi, the majority of the cells acquired a 'feathery' appearance (3+) and by 20 h pi, very few cells remained attached to the glass surface, the majority loosely suspended in the culture medium. This was defined as a (4+) cpe.

In agreement with the growth curve reported by Malsberger and Cerini, Moss and Gravell (1969) reported the presence of virus particles approximately 6 h pi. The particles were randomly distributed throughout the cytoplasm. The authors did not find a specific site of virus replication and virions were found to be associated with tubular structures. No attempt was made to identify them. A similar observation was reported by Dales (1963) for reovirus type 3 in infected L cells. This and other observations led Moss and Gravell to tentatively assign IPN virus with the Reoviridae, which later proved to be erroneous.

The growth curves of IPN virus in BF-2, AS and FHM cells were similar to that of RTG-2 cells. Kelly and Loh (1975) showed that at 22°C, VR-299 IPN virus replicated in SWT cells with a latent period of between 3 and 6 h; virus synthesis was completed by 24 h pi. At 16°C, virus multiplication was slower, the latent period lasted for 12 h and maximum titre was not attained until about 36 h pi. The virus failed to replicate at 30°C, at which temperature the SWT cells have been found to grow more readily than at 16°C.

Virus infection resulted in the inhibition of cellular DNA synthesis in RTG-2 cells (Nicholson, 1971), BF-2 (Argot and Malsberger, 1972), and SWT cells (Kelly and Loh, 1975). Inhibition of DNA synthesis occurred as early as 2 h pi in SWT cells, and 4 to 5 h pi in RTG-2 cells. Total RNA synthesis increased within 2 h pi in SWT cells reaching its maximum by 8 h pi. This increase in RNA synthesis coincided with the exponential production of virus (Kelly and Loh, 1975). Increase in RNA synthesis as a result of viral infection has been shown in RTG-2 (Nicholson. 1971) and BF-2 cells (Argot and Malsberger, 1972).

9. Structure of IPN virus particle

Negative staining of IPN virus revealed naked (nonenveloped) isometric particles with icosahedral symmetry (Moss and Gravell, 1969; Kelly and Loh, 1972; Cohen et al., 1973). Initial studies reported particle diameters of 18.5 and 29 nm (Cerini and Malsberger, 1965). It was therefore thought to be a Picornavirus (Melnick, 1971). Later studies showed the virus size to be 57 nm (Lightner and Post, 1969). In thin sections of infected cells, the particle size was reported to be 65 nm (Wolf and Quimby, 1971) and 55 nm (Moss and Gravell, 1968, 1969; Ball et al., 1971). On the basis of these electron microscopical studies, its size and morphology were similar to those of the reovirus (Moss and Gravell, 1969). That IPN virus may be a reovirus was further substantiated when it was shown that its RNA may be double-stranded (Argot, 1969). More recent studies indicated that IPN virus does not belong to either the Picornavirus or the Reoviridae (Kelly and Loh, 1972; Cohen et al., 1973; Macdonald and Yamamoto, 1977; Dobos, 1976), since the inner capsid structure of the reovirus is lacking in IPN virus. The size of purified IPN virions was reported to be 74 nm by Kelly and Loh (1972), Yamamoto (1974) and about 70 nm by Cohen

et al. (1973). Because of the different sizes reported, it was therefore thought that isolates of IPN virus may be different 'strains'. It seems unlikely that strain differences could account for the conflicting results. Thus, either Cerini and Malsberger did not observe IPN virus particles or they had actually observed artifacts due to their purification procedures. Indeed, their electron photomicrographs revealed very few particles and they were poorly resolved and indistinct. Electron photomicrographs of negatively-stained IPN virus show the virus to have 4 structural components per facet edge (Kelly and Loh, 1972; Cohen et al., 1973; Moss and Gravell, 1969), suggesting that the capsid is made up of 92 capsomeres. However, the individual capsomeres were difficult to resolve in negatively-stained preparations.

10. Structure of IPN virus RNA

The nucleic acid of IPN virus was first reported to be RNA in 1964 after a single acridine orange stain of infected cells (Wolf, 1964). The effect of metabolic inhibitors of nucleic acid synthesis in IPN virus-infected cells also indicated the nucleic acid to be RNA. Halogenated deoxyuridines at 0.0001 M concentration had no effect on virus yield, but virus replication was inhibited by 5,6-dichloro-1B-ribofuranosyl-benzimidazole (Malsberger and Cerini, 1965). Actinomycin D was inhibitory at a concentration of 20μg/ml. During viral infection, the newly synthesized RNA in the cytoplasm of infected cells was sensitive to RNase at 0.02% concentration (Nicholson, 1971). The author failed to detect any fluorescent green-staining cytoplasmic inclusion bodies when virus-infected cells were stained with acridine orange. On this basis, Nicholson suggested that the RNA was probably single-stranded. This was in direct contrast to the report by Argot (1969), who observed the appearance of fluorescent green

staining cytoplasmic inclusion bodies in virus-infected RTG-2 cells, albeit infrequently. Such inclusions were detected as early as 6 h pi. No Feulgen-positive viral inclusions were observed (Argot and Malsberger, 1972). Their results suggested that IPN virus RNA may be double-stranded but was not substantiated by the findings of Kelly and Loh (1972). They found the IPN virus RNA to be 16S during sucrose gradient sedimentation, sensitive to pancreatic RNase digestion, resistant to denaturation at 95 to 100°C and to have a non-conplementary base composition. The G:C ratios reported were however very close to one and may not be significantly different. Kelly and Loh (1972) suggested that, though the RNA was probably single-stranded, "it possibly contained a highly ordered secondary structure or tertiary structure possessing a stable configuration similar to transfer RNA". Their evidence however, was not conclusive, with the added possibility that their RNA preparation may have contained contamina ting single-stranded or other RNA species not removed by their purification procedures, and which were non-viral in nature.

More recent data did not substantiate the report of Kelly and Loh (1972). Cohen et al. (1973) found the virus RNA to be 14S on sucrose gradients, to be resistant to hydrolysis by pancreatic RNase in 1 X SSC buffer and to have a complementary base composition (A/U=G/C = 1) indicative of a double-stranded RNA. They found that their RNA preparation exhibited a sharp melting profile with a Tm of 89°C in 0.1 x SSC buffer and after melting it sedimented at a density corresponding to a single-stranded molecule.

Similar experiments were performed by Macdonald and Yamamoto (1977) and Dobos (1976). In both studies, the viral RNA was found to be 14S on sucrose gradients, while at high salt concentration it was resistant

to RNase digestion, but became sensitive at low salt concentration (Macdonald and Yamamoto, 1977). The virus RNA had a density of 1.6 g/cc in cesium sulfate, and the G:C ratio was found to be one. Electron microscopic measurements of the RNA showed it to be double-stranded (Macdonald and Yamamoto, 1977). In addition, upon denaturation in 98% formamide the viral genome sedimented at 24S in a formamide sucrose gradient and became sensitive to RNase digestion (Dobos, 1976). The viral RNA when denatured with 90% DMSO (dimethylsulphoxide) did not revert to the 14S form upon re-centrifugation in aqueous sucrose gradient containing 0.1 M NaCl, but co-sedimented with the 25S segments of similarly denatured reovirus RNA (Dobos, 1976). The authors could detect no single-stranded regions in the viral genome. IPN virus RNA is therefore a double-stranded molecule in its native configuration.

The IPN virus genome has now been shown to contain two double-stranded RNA species by SDS-polyacrylamide gel electrophoresis (Macdonald and Yamamoto, 1977; Dobos, 1976) and constitute 8.7% of the virion mass (Dobos, 1977). The average molecular weight for the two RNA species was estimated to be from 2.3 to 2.6 x 10^6 daltons. IPN virus RNA is therefore not similar nor related to the RNA's of reovirus. Instead, it may be related to the fungal viruses and virus-like particles from fungi (Wood and Bozarth, 1972; Bevan et al., 1973; Herring and Bevan, 1974; Buck and Ratti, 1975). The fungal viruses are, however, smaller in size (22 nm diameter) than IPN virus.

11. The polypeptides of IPN virus capsid

Conflicting data have been reported for the polypeptides of IPN virus by several groups of workers. Loh et al. (1974) were able to resolve 7 polypeptides from SDS-polyacrylamide gel electrophoresis of

solubilized protein from [^{3}H]-labelled amino-acid IPN virus. The protein components ranged from 34,000 to 125,000 daltons. Only two of the polypeptides were major bands constituting 57% and 20% of the total radioactivity with molecular weights of 58,000 and 38,000 respectively. The 125,000 dalton protein represented only 2.5% of the total radioactivity. Cohen et al. (1973), on the other hand, could only resolved 3 protein components by SDS-gel electrophoresis from IPN virions which had been solubilized by treatment with urea and SDS. Their molecular weights were estimated to be 80,000; 50,000 and 30,000 daltons, constituting 3, 68 and 29% of the total radioactivity respectively. A more carful study of their data on the migratory pattern would seem to indicate very little difference between the results of these groups of workers: i.e. the 80,000; 50,000 and 30,000 dalton proteins could correspond to the 125,000; 58,000 and 38,000 dalton proteins detected by Loh et al. The differences in the molecular weights obtained may be due to the different protein standard that were employed by the two groups of workers. It is also possible that, in the data of Loh et al. (1974), contaminants may have been present that contributed to the presence of the numerous minor bands or that these were the result of a breakdown of the major protein bands. Three classes of IPN virus proteins have recently been reported by Dobos (1976). There were designated as large (80-120,000 daltons), medium (65-50,000 daltons) and small (35-30,000 daltons). There is thus general agreement that IPN virus contains at least 2 polypeptides in its capsid, although the possibility of a third cannot be ruled out. It is interesting that the 80,000 dalton protein was not consistently present in SDS gels (Cohen et al., 1973). To date, the nature of the polypeptides of other IPN virus isolates has not been determined.

12. Autointerference by IPN virus in infected cells

In many viral systems, serial undiluted passages of virus lead to the accumulation of defective interfering (DI) particles and the development of homologous interference. IPN virus was first reported to be able to cause autointerference by Malsberger and Cerini in 1963. They showed that serial passages of undiluted virus caused an overall reduction of the virus yield per cell. In a study by Nicholson and Dunn (1974), infection of RTG-2 and AS cell cultures with undiluted stock of an IPN virus isolate (Dry Mills) obtained by several passages at high input multiplicity resulted in little, if any, cpe and resulted in reduced virus yields. When susceptible cells were infected with a diluted stock virus, extensive cpe occurred. The infected cultures yielded a virus titre of $10^{8.4}$ $TCID_{50}$/ml, while undiluted stock virus yielded less. Pretreatment of cell cultures with interfering virus inhibited the replication of homologous infective virus, but not unrelated virus such as Infectious Hematopoetic Necrosis (IHN) virus and the Bluegill Myxovirus (BMV). Interference due to interferon was experimentally ruled out (Nicholson and Dunn, 1974). The interference was abolished by UV irradiation and one cycle of freezing-and thawing of high input multiplicity passaged IPN virus. Interference in the isolation of IPN virus from carrier fish was demonstrated by Nicholson and Dexter (1975). Cultures of RTG-2 cells, when inoculated with concentrated tissue homogenates of suspected IPN virus carrier showed little, if any cpe. However, when cells were infected with a relatively high dilution of the same tissue homogenates, cpe was evident as early as 24 h pi, with high yield of IPN virus.

Thus, the available data indicate that some type of interference of IPN virus replication can occur when undiluted tissue homogenates or

virus suspensions are used to infect cell cultures. Further studies are required to demonstrate directly the existence of DI particles in interfering IPN virus stocks. To achieve this the ratio of infectious virus to actual number of viral particles must be established. Since IPN virus has been shown to lack hemagglutinating or hemadsorbing properties (Malsberger and Cerini, 1963), detection of DI particles by serological means is not feasible.

13. RNA polymerase (Transcriptase) activity in IPN virus

IPN virions have been shown to contain an RNA dependent RNA polymerase which is capable of catalyzing the synthesis of single-stranded RNA from double-stranded RNA genome (Cohen, 1975). The location of the polymerase in the virion is not yet known. Purified IPN virus was found to be enzymatically active without any treatment. This is in contrast to reovirus whose outer capsid shell must be partially or completely removed from the RNA polymerase to be activated (Borsa and Graham, 1968). It is interesting to note that other Diplornaviruses having a single-shelled capsid (e.g. Cytoplasmic polyhedrosis virus, Penicillin stoloniferon virus) also exhibit a polymerase activity (Lewandowski et al., 1969; Lapierre et al., 1971) which, like IPN virus requires no activation.

All the evidence to date suggests that IPN virus probably belongs to a new group of possibly closely related viruses that are yet unclassified. Such viruses may include the Infectious Bursal Disease virus (IBDV) of chickens (Nick et al., 1976). This agent, like IPN virus contains RNA in two discrete fragments, but with a slightly smaller particle of 64 nm in diameter and a buoyant density of 1.32 g/cc, which is very close to the value reported for IPN virus. IBDV is also morphologically similar to IPN virus in negatively stained preparations.

14. Purification of IPN virus

Two essential requirements must be met in order to study a virus; firstly, a cell culture system must be available not only for the propagation of the virus but also for quantitative assays of virus infectivity and secondly, a purification method must be available in order that the virus can be concentrated and purified with minimal loss of infectivity.

Several procedures have been reported for the purification of IPN virus from infected cells and infected cell culture medium. These procedures include (1) extraction of sonicated cell-fluid by Freon followed by high speed centrifugation to pellet the virus (Moss and Gravell, 1969); (2) low speed centrifugation of the cell-fluid sonicate followed by two cycles of centrifugation at high speed (78,000 g) to pellet the virus from the medium (Kelly and Loh, 1972) and (3) extraction of the virus-cell mixture with freon followed by PEG precipitation of the virus (Cohen et al., 1973). In all cases a final purification step was included in which the virus was banded either in a potassium tartrate gradient (Moss and Gravell, 1969), twice in a CsCl gradient centrifugation (Kelly and Loh, 1972), or once in a sucrose gradient followed by a CsCl gradient centrifugation (Cohen et al., 1973). With regard to the efficiency of these methods, the recovery of virus infectivity has not been quantitatively determined nor clearly demonstrated. Cohen et al., (1973) published figures that showed good recovery of infectious virus in their Freon and PEG concentrates. The major problem encountered was the liberation of the 50% or more of the virus that remained associated with the cell. The author found that freezing and thawing, treatment with a high frequency sound, treatment with deoxycholate, NP40 and Triton X-100 (both non-ionic

detergents) or chymotrypsin was not effective in liberating the virus from the cell. The most satisfactory method was to treat the cell pellet with Freon 113 (Trifluorotrichloroethane). They found that the virus carried in the supernatant could be precipitated and concentrated with PEG.

The use of fluorocarbons such as Freon 113 has been reported for the purification of animal viruses and plant viruses such as tobacco mosaic virus (TMV) and ringspot viruses (Porter, 1956) and cytoplasmic polyhedrosis virus of the silk-worm (Lewandowski et al., 1969). The basis of the selectivity of fluorocarbon treatment is unknown. Viruses that contain lipids in general are susceptible to fluorocarbon denaturation (Hamparian et al., 1958; Gessler et al., 1956). It appears that fluorocarbon extracts lipids and denatures proteins of certain sizes. For example, the complement fixing antigens of coxsackie and poliomyelitis viruses were unaffected by the treatment, while those of mumps, influenza V and S antigens were precipitated after two fluorocarbon treatment. Both the latter viruses have a relatively high lipid content. Brown and Cartright (1960) showed that while the infectivity of foot-and-mouth disease virus (FMDV) was unaffected, the complement fixing titre was reduced by 10-20% after fluorocarbon treatment. Apparently a 7 nm non-infective viral component was completely denatured, while the 20 nm infective virus particle remained unaffected. Purified viruses tend to be inactivated by fluorocarbon treatment. This would seem to indicate that the presence of some impurities may serve to protect the virus from denaturation (Phillipson, 1967).

PEG was initially employed in combination with dextran-sulfate as a method for concentrating and purifying viruses (Albertsson, 1960;

Albertsson and Frick, 1960; Phillipson et al., 1960; Norby and Albertsson, 1960; Frick, 1961). It was subsequently shown that PEG alone could precipitate plant viruses (Herbert, 1963; Venekamp and Mosch, 1964a and 1964b). This was later confirmed and extended to bacteriophage T_4 by Leberman (1966). PEG alone did not have the disadvantage of contamination of the concentrated virus by dextran-sulfate which required special procedures for its removal. Yamamoto et al. (1970) found that an aqueous solution of PEG (2-10%) and NaCl (0.5 M) enabled bacteriophages either to be pelleted by low speed centrifugation or to be settled out and further concentrated by pressure filtration. The bacteriophages tested were Lambda, T_4, T_7, P22, fd, ØX174 and R17. All were efficiently removed from solution by centrifugation with yields as high as 95% recovered. Low concentrations of PEG was found to selectively precipitate asymmetric particles, thereby allowing a separation from more symmetrical particles (Yamamoto et al., 1970). A 100 fold concentration of Vesicular stomatitis virus (VSV) by this method was reported by McSharry and Benzinger (1970). The virus could be separated from much of the host protein and phospholipid, with 97% recovery of infectivity from the PEG pellet. Low titre lysates could also be effectively treated with PEG and NaCl (Yamamoto et al., 1970). The mechanism by which virus particles become rapidly sedimenting in PEG-containing solutions is unknown.

15. Objectives of this study

The primary object of this study was to determine whether there are fundamental differences between VR-299 IPN virus, Jasper IPN virus and 8 other isolates of IPN virus with respect to morphology, particle size, buoyant density in CsCl and polypeptide composition or purified virions.

The experimental approach of this study was threefold:

(a) To establish the use of the CHSE-214 cell line for virus propagation, production and titration by plaque assay.

(b) To establish a standard procedure for the purification, concentration and recovery of infectious IPN virus. The effectiveness of Freon and PEG in concentrating various viruses discussed above indicated that these methods could be applied to concentrate IPN virus from infected cells and infected cell-culture media.

(c) To compare 10 isolates of IPN virus by electron microscopy, CsCl gradient centrifugation and SDS-polyacrylamide gel electrophoresis of purified IPN virus proteins.

MATERIALS AND METHODS

1. Virus Isolates

Reference IPN virus (VR-299) was obtained from the American Type Culture Collection (ATCC, Rockville, Md., U.S.A.). The Jasper isolate was obtained from Dr. T. Yamamoto (Department of Microbiology University of Alberta). The following IPN virus isolates were the kind gift of Dr. K. Wolf (Eastern Fish and Disease Laboratory, Kearnysville, W. Va., U.S.A.): Powder Mill (New Hampshire), Buhl (Idaho), Reno (Navada), West Buxton (Maine), Bonnamy (France) and d'Honnincthun (France). The Fall River (FR) and Western IPN virus isolates were obtained from Dr. J.L. Fryer (Department of Microbiology Oregon State University, Corvallis, Oregon, U.S.A.).

2. Cell Culture

Two cell lines were employed during this study. The Chinook salmon embryo (CHSE-214) cell line was obtained from Dr. J.L. Fryer (Oregon State University, Corvallis, Oregon) and the Rainbow trout gonad (RTG-2) cell line was obtained from the American Type Culture Collection (ATCC-CCL-55).

The virus isolates were continuously propagated in CHSE-214 cells. In some specific studies, the RTG-2 cell line was used. The following isolates VR-299, Jasper and FR were plaque-purified three consecutive times in CHSE-214 cells. Stock virus was prepared by inoculating a confluent monolayer of CHSE-214 cells, which were contained in a 3 oz. prescription bottle with 0.1 ml of the required virus isolate. The infected cell culture was incubated at 18°C and

maintained in MEM plus 5% FCS until maximum cpe occurred, normally within three days. This was used as the stock virus for each experiment, and was stored at 4°C to be used within a short period of time, otherwise samples were frozen at -65°C.

3. Cell Culture Media

Eagle's MEM (GIBCO, Grand Island, N.Y.) containing 100 IU/ml of penicillin (GIBCO, Grand Island, N.Y.), 100 µg/ml of streptomycin (GIBCO, Grand Island, N.Y.) and 5% FCS (GIBCO, Grand Island, N.Y.) plus 0.22% sodium bicarbonate was used throughout for cell culture maintenance in Roux bottles. To maintain cell cultures in 60 mm tissue culture dishes (Falcon Plastics, Oxnard, Ca., 93030 U.S.A.) for plaque assays, Eagle's MEM with 5% FCS, 0.08% sodium bicarbonate, 14 mM HEPES (Calbiochem, L.A., Ca., U.S.A.), penicillin (100 IU/ml) and streptomycin (100 µg/ml) was routinely used as the overlay medium.

4. Cell Culture Maintenance

RTG-2 and CHSE-214 cells were routinely maintained in Roux bottles. To transfer cells, bottles containing confluent monolayers of cells were treated with 10 ml of 0.2% trypsin at room temperature for a sufficient length of time (normally 5-10 min) to detach the cells from the glass. The cell suspension was centrifuged at 2000 rev/min in a clinical centrifuge (O.H. Johns Scientific, Toronto) for 10 min. The supernatant was discarded and the cells were resuspended from the pellet in sufficient growth medium so that each Roux bottle to be seeded received an aliquot of 100 ml of cell suspension at a cell concentration of 2-3 x 10^5 cells/ml. A single Roux bottle of confluent cells was normally reseeded into 3 Roux bottles by this method. A

complete monolayer was usually formed within 2-3 days, and can be maintained for 4 to 6 weeks before being used for virus infection or for reseeding into fresh Roux bottles. No medium change was required during the whole period. It was found that RTG-2 cells could be maintained for about 3 weeks while CHSE-214 cells could be maintained up to 6 weeks before reseeding.

5. Propagation of Virus

For routine production of virus, 5 Roux bottles of confluent CHSE-214 cells were used. Prior to infection of cells with virus, the cell culture medium was removed. One ml of a 1/1000 dilution of a stock virus suspension containing 1 to 3 x 10^8 PFU/ml was added to the cell monolayer. Adsorption was allowed to proceed for 1 hr and each bottle containing infected cells was gently rotated at 15 min. intervals; then 100 ml of MEM growth medium plus 5% FCS was added to each bottle. Infected cell cultures were then incubated at 18°C until complete cpe was evident, usually in three days.

6. Purification and Concentration of Virus

Infected cells and medium were removed from Roux bottles and centrifuged at 5000 rev/min (4920 g) in a Sorvall RC-2B centrifuge using the GSA rotor (Ivan Sorvall Inc., Norwalk, Conn.) for 15 min. The supernatant was removed and treated with PEG (molecular weight 20,000, J.T. Baker Chemicals, Phillipsburg, N.J.). The cells from the pellet were resuspended in 2 to 3 ml Tris-HCl buffer, pH 7.3 containing 0.025 M Tris (Fisher Scientific Co., Fair Lawn, N.J.), 0.001 M EDTA (Matheson, Coleman and Bell, Norwood, Ohio) and 0.1 M

NaCl (J.T. Baker Chemicals, Phillipsburg, N.J.). The cell resuspension was then treated with Freon 113 (Dupont, Maitland, Ontario.).

(a) PEG precipitation of culture medium containing virus.

The medium containing virus was made into a mixture containing 2.2% NaCl and 5% PEG. It was stirred at 4°C for 2 to 4 h. and precipitable material was sedimented by centrifugation at 10,000 rev/min (16,300 g) for 1 h 15 min in a Sorvall RC-2B centrifuge using the GSA rotor. The supernatant was discarded and the pelleted material was resuspended in 2-3 ml of Tris HCl buffer, vortexed gently and centrifuged at 1,000 rev/min to pellet the PEG. The supernatant was removed and the virus further purified by CsCl gradient centrifugation.

(b) Freon extraction of virus cell-mixture.

The resuspended cells were mixed with an equal volume of Freon and vortexed vigorously for 2-5 min. After centrifugation at 2,000 rev/min in a clinical centrifuge (O. H. Johns Scientific, Toronto), the upper aqueous phase containing the virus was removed with a Pasteur pipette and stored at 4°C prior to further purification by CsCl gradient centrifugation. The remaining interphase layer was re-extracted as described previously.

(c) CsCl gradient centrifugation of IPN virus.

The PEG concentrated and Freon extracted virus samples were treated with RNase (20 µg/ml) and layered over a stepwise gradient consisting of 1.5 ml of 40% CsCl,

1,0 ml of 30% CsCl and 0.5 ml of 20% CsCl. Centrifugation was allowed to proceed for 16 h at 4°C in a Beckman ultracentrifuge (Model L265B) using the SW 50.1 rotor at 35,000 rev/min. (114,000g). The virus band which was clearly evident was withdrawn from the top of the centrifuge tube with a syringe. Virus samples were pooled and dialyzed overnight against Tris-HCl buffer, pH 7.3 to remove the CsCl. The dialyzed sample was then layered onto a CsCl gradient prepared as described above and centrifuged at 35,000 rev/min for 3 h at 4°C. The virus in the form of a visible band at the end of this period was removed as previously described and dialyzed against Tris-HCl buffer at 4°C. Of the total protein found in the culture supernatant and Freon extract, only 0.01% and 1.4% was respectively recovered in the virus band from the second CsCl gradient described above.

(d) Sucrose gradient centrifugation of IPN virus.

Sucrose (Fisher Scientific Co., Fair Lawn, N.J.) gradient solutions of 15 and 30% were prepared in Tris-HCl buffer, pH 7.3 [^{3}H]-Guanine (Amersham/Searle, Arlington Heights, Illinois) labelled virus was layered on a 15 to 30% stepwise gradient and centrifuged for 1 h 15 min at 30,000 rev/min (154,000 g) in the Spinco SW 40 Tl rotor at 4°C. Drops were collected from the bottom, and radioactivity was counted directly in Bray's scintillation fluid (Bray, 1960). Radioactive virus fractions were pooled and dialyzed against Tris-HCl buffer.

7. Plaque Assay and Limiting Dilution Assay

The plaque assay method was employed to monitor virus infectivity. Confluent cultures of CHSE-214 cells in 60 mm tissue culture dishes were inoculated with 0.1 ml of virus diluted in Tris-HCl buffer, pH 7.3. Adsorption was allowed to proceed for 1 hr at 23°C. The culture dishes were then placed on a tray of ice to allow rapid cooling. Each culture dish was then overlayed with 8-10 ml of an overlay mixture containing equal volumes of the overlay medium (MEM with 0.08% sodium bicarbonate, 14mM HEPES, penicillin, 100 IU/ml and streptomycin, 100 µg/ml) and 1% Agarose (Seakem mci Biomedical Co., Springfield, N.J.) solution containing 20% FCS. The final mixture contained 0.5% Agarose and 10% FCS. The Agarose solution was brought to a boil and then cooled to 43°C. FCS was then added; it was then mixed with the overlay medium and used immediately. When the Agarose-overlay had hardened (3-5 min), culture dishes were incubated at 18°C for 3-4 days. The cell culture was then fixed with 30% formaldehyde (J.T. Baker Chemical Co., Phillipsburg, N.J.) for at least 1 h after which time the Agarose-overlay was removed and the cells stained with crystal violet and the plaques enumerated and observed with the inverted light microscope.

For certain specific experiments, virus titre was determined by the Limiting Dilution Assay ($TCID_{50}$) method. One tenth ml of log dilutions of a virus suspension were inoculated into 4 culture tubes of CHSE-214 cells. The infected cell cultures were maintained with MEM growth medium plus 5% FCS and incubated at 18°C. Cell cultures were examined daily for cpe with an inverted light microscope for a period

of 2 weeks. The $TCID_{50}$ was calculated according to the method of Reed and Muench (1938).

8. Radioactive Labelling of IPN Virus RNA

To prepare radioactive virus, 0.3 ml of a fresh stock virus was made up to 1 ml with Tris-HCl buffer, and added to each Roux bottle after removal of the old medium. Adsorption was allowed to proceed for 1 h at room temperature; then 100 ml of MEM growth medium plus 5% FCS and 1 µCi/ml of [^{3}H]-Guanine (6.8 Ci mmole) was added. Incubation was at 18°C for 24-48 h, or until maximum cpe was evident.

9. Analysis of IPN Virus following CsCl Gradient Centrifugation

The buoyant density of IPN virus was determined in the following manner. Radioactive virus purified by a sucrose gradient centrifugation described above was layered on a CsCl gradient as previously described and centrifuged for 24 h in the Spinco SW 50.1 rotor at 35,000 rev/min at 4°C. Drops were collected from the bottom and kept on ice until the refractive index (R.I.) was determined using an Erma refractometer (Erma Optical Works, Tokyo, Japan). The R.I. was corrected by subtraction of 0.002 units from each reading to compensate for the contribution by Tris-HCl buffer. Pycnometric readings were also obtained with micro pipettes. Each fraction was then precipitated with 10% trichloroacetic acid (J.T. Baker Chemical Co., Phillipsburg, N.J.). Yeast RNA was added as carrier for precipitation. Incubation was for 30 min at 4°C. Precipitable samples were filtered through Whatman GF/A glass fibre filters; and the filters were dried and counted in toluene-POPOP-PPO scintillation fluid (Kent Laboratories Ltd.,Van.).

To relate the absorbance at 260 nm with viral infectivity and buoyant density in CsCl, the following experiment was performed. Virus purified by a second CsCl gradient centrifugation was layered on a 20 to 40% CsCl gradient and centrifuged for 24 h in the Spinco SW 50.1 rotor at 35,000 rev/min at 4°C in the Beckman ultracentrifuge (Model L2-65B). Fourteen drop fractions were collected from the bottom of the tube. The absorbance at 260 nm of each fraction was determined with a Gilford spectrophotometer 240 (Gilford Instrument Inc., Oberlin, Ohio). The R.I. was determined for each fraction as previously described. Infectivity of each fraction was determined by the plaque assay method.

10. Electron Microscopic Observation of purified IPN Virus

Negative staining of IPN virus was carried out as follows: Purified virus in Tris-HCl buffer was mixed with a suitable volume of 3% PTA at pH 7.2 containing latex particles of 88 nm in diameter. The mixture of virus, latex and PTA was allowed to adsorb onto the grid for 1 min. After draining off excess fluid, the grid allowed to dry and immediately examined in a Phillips EM 200 electron microscope operated with a 60Kv beam and double condenser illumination. Copper (200 mesh) grids (Ted Pella Co., Tustin, California) were coated with a film of formvar and stabilized with evaporated carbon. Photographs were taken at instrumental magnification of 11,200 times unless otherwise stated, on Kodak 35 mm fine grain positive film.

11. Particle Counts of Virus and Latex Suspensions

Particle counts of virus and latex suspensions were performed according to the lowered drop method of Pinteric and Taylor (1962) with

some slight modifications. A known concentration of newly purified virus was initially fixed with 0.3% glutaraldehyde (prepared with 0.1 M phosphate buffer, pH 7.2) final concentration, for 30 min at 4°C and then allowed to mix with a calibrated suspension of latex particles of 264 nm diameter (The Dow Chemical Co., Midland, Michigan). Several drops of the mixture were then dispensed (with a 5 µl pipette) on top of a formvar film floating on the surface of a freshly prepared ammonium acetate/ammonium carbonate solution, pH 7.3. The solution mixture contained 0.01 M ammonium acetate instead of the 0.15 M concentration employed by Pinteric and Taylor (1972). The drops were allowed to dialyze for 1 h at room temperature. The level of the buffer was then gently lowered until the drops were centered on titanium grids (Ted Pella Co., Tustin, California) resting on a piece of sintered glass. The grids were then dried in a lid-covered petri-dish containing pellets of NaOH (J.T. Baker Chemical Co., Phillipsburg, N.J.), shadowed with platinum-palladium, stabilized with evaporated carbon and examined directly in the electron microscope. The concentration of virus particles in the shadowed preparation was estimated by counting the latex and virus particles in randomly selected fields. The concentration of virus particles was calculated by the following equation.

$$\frac{\text{No. of virus particles per given area}}{\text{No. of latex particles in same area}} \times \text{Concentration of latex particles/ml.}$$

12. Absorption Spectrum of IPN Virus

The absorption spectrum of IPN virus from 200 to 300 nm wavelength was determined with the Gilford Spectrophotometer using IPN virus purified from a second CsCl gradient centrifugation. The

$A_{260/280}$ ratio was also determined simultaneously.

13. Viral Protein Determination

To estimate the amount of viral protein, a Lowry protein determination (Lowry et al. 1951) was carried out with a suspension of purified virus. Crystalline bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) was used as a standard. Readings were obtained colorimetrically on a Beckman DB-G grating spectrophotometer at 500 nm wavelength. One ml of purified virus suspension normally contained 240 µg protein and is equivalent to 1.3×10^{10} PFU.

14. SDS-polyacrylamide Gel Electrophoresis of IPN Virus Proteins

SDS-slab-gel electrophoresis has recently been extensively employed by workers in the field of phage protein biosynthesis (Studier, 1972, 1973; Lengyel et al. 1973; O'Farrell and Gold, 1973), in the analysis of herpes virus proteins (Honess and Roizman, 1973), in the identification of coliphage P2, P4 and the bacteria E. coli proteins (Knight, 1975), in the identification of the components of the histidine transport system in S. typhimurium (Ames, 1974) and in the study of bacterial membrane proteins (Ames, 1974).

The reliability of molecular weight estimations by this system has been documented by Neville (1971) and Weber and Osborn (1969). Approximately 20 µg of protein per sample was found to give optimal resolution and 0.2 µg of protein was found to be visible in a single band (Ames, 1974). The difficulty in obtaining large amounts of virus prompted the use of this technique for analyzing the proteins of several isolates of IPN virus in this study.

SDS-gel electrophoresis of IPN virions was performed according to the method of Laemmli (1970). The acrylamide (Matheson, Coleman and Bell, Norwood, Ohio) was purified according to the method of Loening (1967). Gels containing 5% (stacking) and 12% (separating) acrylamide were prepared from a stock solution containing 30% by weight of acrylamide and 0.85% by weight of N, N'-bis-methylene acrylamide (Matheson, Coleman and Bell, Norwood, Ohio).

(a) Preparation of separating and stacking gels

The separating gel (Laemmli, 1970) was prepared first and contained 0.375 M Tris-HCl(pH 8.8) and 0.1% SDS (Sigma Chemical Co., St. Louis, Mo.). Polymerization was allowed to proceed by the addition of 0.08% by volume of N,N,N',N'-tetramethyl-ethylenediamine (TEMED, Eastman Kodak Co., Rochester, N.Y.) and ammonium persulfate (Allied Chemical, Morristown, N.J.), Tris was added last. The solution was degassed under vacuum and poured immediately into the gel apparatus.

The stacking gel (Laemmli, 1970) of 5% acrylamide contained 0.125 M Tris-HCl (pH 6.8) and 0.1% SDS, and was polymerized chemically in the same manner as for the separating gel.

The polyacrylamide gel solution mixture was made up from stock solutions described in [H] as follows:

Reagents	Volume (ml) 5% gel	8% gel	12.5% gel
M Tris pH 8.8	-------	30	30
M Tris pH 6.8	2.50	------	------
10% SDS	0.20	0.80	0.80
30% acrylamide 0.85 % Bis-acrylamide	3.32	21.26	33.30
TEMED	10µl	60µl	60µl
Distilled water	13.80	20.80	15.50
10% ammonium persulfate	0.10	0.60	0.60

(b) Preparation of electrode buffer

The electrode buffer (pH 8.3) contained 0.025 M Tris and 0.192 M Glycine (Amino-acetic acid, Fisher Scientific Co., Fair Lawn, N.J.) and 0.1% SDS. The buffer solution was made up as follows:

Reagent	Volume (ml)
X 10 Tris-glycine	200
10% SDS	20
Distilled water	1780
Total volume	2000

(c) Denaturation of purified virus with SDS and 2-mercaptoethanol

Purified virus was first dialyzed against distilled water

to remove all salts, and then lyophilized under vacuum at -70°C. The dried virus sample was resuspended in a solution containing in final concentration 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol (Fisher Scientific Co., Fair Lawn, N.J.) and 0.001% bromophenol blue (Matheson, Coleman and Bell, Norwood, Ohio). The virus proteins were completely dissociated by immersing the samples in boiling water for 2 min.(Maizel, 1969).

(d) Procedure for slab gel apparatus

The separating and stacking gels were dispensed into a slab gel apparatus of dimensions 28 cm x 1.5 mm (Hoeffer Scientific Instruments, San Franscisco, Cal.). Separating gel was poured first, and overlayed with a thin film of 1-butanol (J.T. Baker Chemical Co., Phillipsburg, N.J.). After polymerization by addition of TEMED and ammonium persulfate (10-15 min) the top of the gel was rinsed with distilled water containing 0.1% SDS and stacking gel was poured immediately. The comb with the slots was inserted into the stacking gel before polymerization occurred. Air bubbles were carefully removed by gently moving the comb up and down. After the polymerization, the comb was removed, and the slots were overlayed with the electrode buffer (running buffer). The protein samples containing approximately 30-50 µg were then dispensed into each slot of the stacking gel. Electrophoresis was carried out with an initial current of 5 mAmp until the

tracking dye was concentrated at the interphase between the separating and stacking gel. The current was then increased to 12.5 mAmp until the bromophenol blue reached the bottom of the gel, at which time the slab gel was removed, fixed and stained.

(e) Procedure for the fixation and staining of proteins in gels.

Proteins in the gel were fixed and stained according to the method of Fairbanks et al (1971). The gel was fixed in a solution containing 25% 2-propanol (isopropanol, J.T. Baker Chemical Co., Phillipsburg, N.J.), 10% acetic acid (J.T. Baker Chemical Co.) and 0.05% coomassie blue (Searle Diagnostic, High Wycombe, Bucks. England) for 12 h. This was followed by one change with a solution containing 25% 2-propanol, 10% acetic acid and then several changes with a solution containing 10% 2-propanol and 10% acetic acid until the gel was completely destained. The gel containing the stained protein bands was photographed in the wet state with a red filter, and 35 mm high contrast film (Kodak). Gels were either sliced into individual strips or preserved by drying on Whatman filter paper under vacuum at 100°C.

(f) Densitometer tracings of coomassie blue stained gels

The slab gels were sliced lengthwise into appropriate strips; each strip contained the protein bands of individual samples. The stained bands were then scanned at 620 nm in a

Gilford spectrophotometer equipped with a linear transport scanner (Varicord Model 43, Photovolt corporation N.Y. City N.Y.).

(g) Standard molecular weight marker proteins

The following enzymes were employed as molecular weight standards with the molecular weights given by Weber and Osborn (1969).

Protein	Molecular weight (daltons)	Manufacturer
Bovine serum albumin (BSA)	68,000	Sigma Chemical Co.
Y-globulin (H chain)	50,000	Sigma Chemical Co.
Ovalbumin	43,000	Sigma Chemical Co.
Chymotrypsinogen	25,000	Calbiochem
β-lactoglobulin	18,000	Calbiochem
Lysozyme	14,300	Sigma Chemical Co.
Ribonnuclease	13,700	Calbiochem

(h) Stock solutions for gel electrophoresis

The following stock solutions were prepared:

30% acrylamide, 0.85% Bis-acrylamide (W/V)

10% SDS

TEMED undiluted

10% ammonium persulfate prepared fresh for each experiment

1 M Tris pH 6.8 and pH 8.8

X 10 Tris-glycine (30.3 g Tris; 144 g glycine in 1 litre distilled water).

RESULTS

1. Cytopathic Effect caused by IPN Virus in CHSE-214 and RTG-2 Cells

In IPN virus-infected CHSE-214 cells, cpe was usually observed 48 h after infection. The characteristic epithelial morphology (Fig 1) was no longer evident as cells rounded up, became pycnotic and underwent degeneration (Fig 2). No inclusions were detectable in cells stained with hematoxylin-eosin. When maximum cpe was attained, the majority of cells became detached and contact inhibition was no longer observed between cells that remained attached to the glass surface. When a plaque assay was performed with confluent cultures, no plaques were evident in the control uninfected cells (Fig 3) while distinct plaques were apparent 3 to 4 days after infection (Fig 4). The diameter of individual plaques ranged from 0.1 to 0.4 cm. The majority of the plaques were homogeneous in size. The variation in plaque size was always present even though the infecting virus had been plaque-purified three consecutive times. Each plaque was evident as a circular clearing with a few cells dispersed throughout the zone of clearing.

In IPN virus-infected RTG-2 cells, the cpe observed was different from that observed for CHSE-214 cells. The characteristic features were cell shrinkage with resultant formation of filamentous strands (Fig 6) in contrast to the appearance of normal uninfected cells (Fig 5). Unlike CHSE-214 cells, there was no rounding up of cells, instead the cells took on a feathery appearance. No inclusions were detectable when cells were stained with hematoxylin-eosin. Plaques formed in RTG-2 cells revealed the remains of infected cells within the zone of clearing (Fig 8). A fibrillar and strandy appearance was a characteristic feature around

the edges of the plaques. The majority of the plaques were homogeneous in size. No plaques were evident in uninfected RTG-2 cells (Fig 7).

The above observations indicated that CHSE-214 cells were sensitive to IPN virus infection and could be used in conjunction with RTG-2 cells or alone as an alternative cell line for the diagnosis and growth of IPN virus.

Figure 1

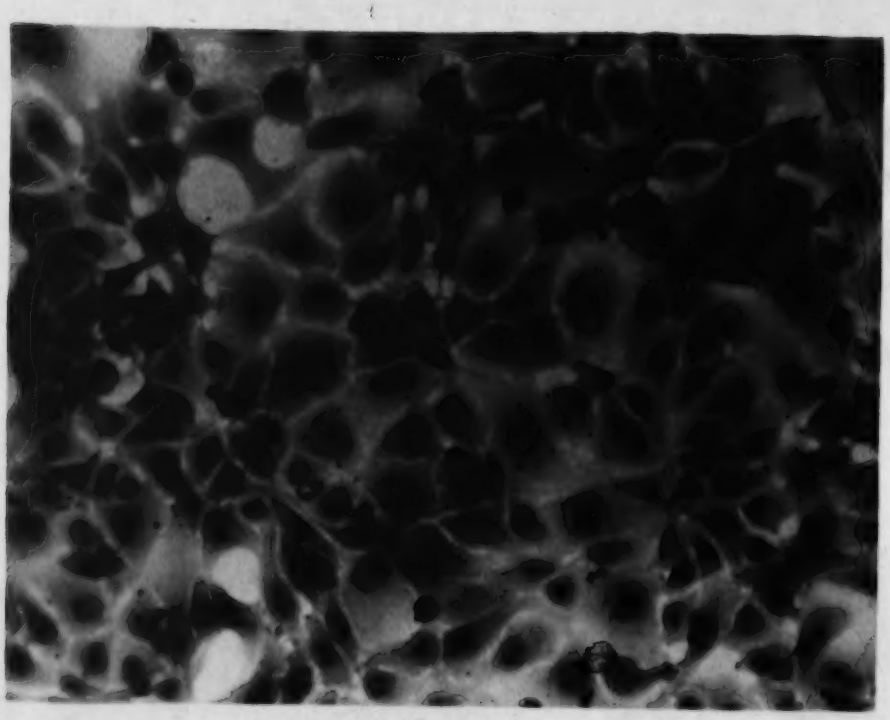
CHSE-214 Cells stained with Hematoxylin-Eosin

Confluent cultures of CHSE-214 cells were grown in Leighton tubes at 23°C. The cells were fixed with methanol and stained with hematoxylin-eosin. The characteristic epithelial morphology is evident. (X 250)

Figure 2

CHSE-214 Cells infected with VR-299 IPN Virus and stained with Hematoxylin-Eosin

Confluent monolayer of CHSE-214 cells in Leighton tubes were infected with 0.1 ml of 1/1000 dilution of stock virus. At 3 days after infection, when cpe was evident, cells were fixed with methanol and stained with hematoxylin-eosin. A characteristic rounding up of cells is evident.
(X 250)



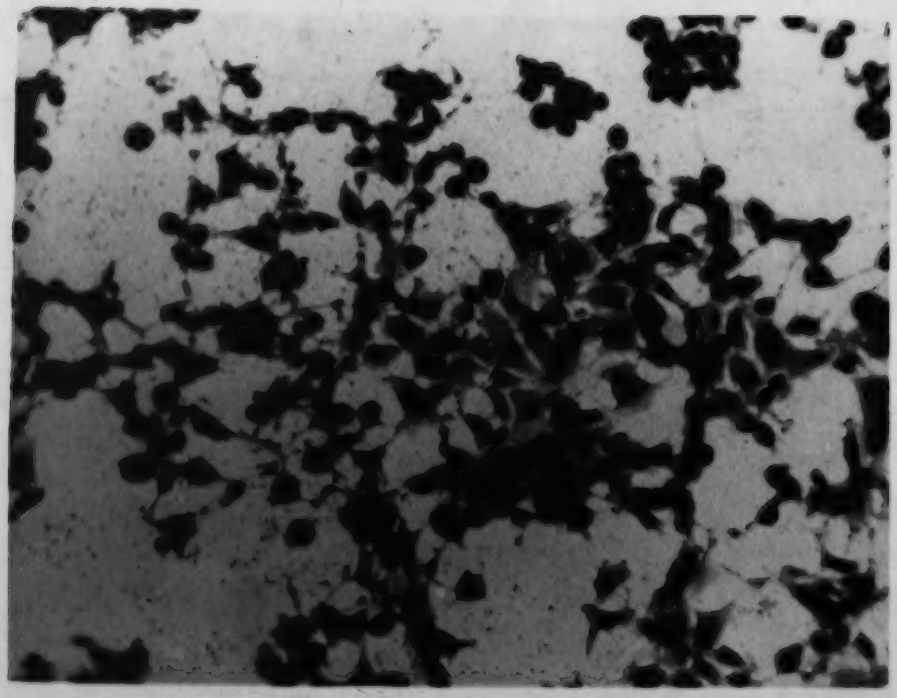


Figure 3

CHSE-214 Cells stained with Crystal Violet

CHSE-214 cells were grown in 60 mm tissue culture dishes until confluent and was then overlayed with MEM overlay medium as described in materials and methods and incubated at 18°C for 3 days. Cells were then fixed with 30% formaldehyde and stained with crystal violet.
(X 250)

Figure 4

Plaque formed by infection of CHSE-214 Cells with VR-299 IPN Virus

CHSE-214 cells were grown in 60 mm tissue culture dishes until confluent. Cells were then infected with 0.1 ml of a virus suspension and overlayed with MEM overlay medium as described in materials and methods. Cells were fixed at 3 days after infection with 30% formaldehyde and stained with crystal violet. Infected rounded cells are evident within the zone of clearing.
(X 150)

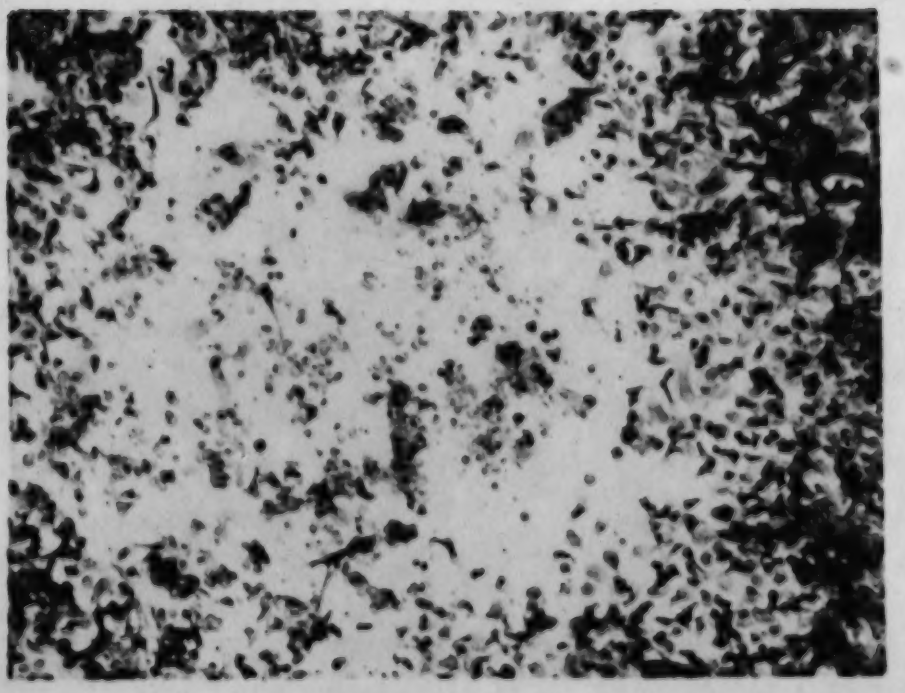


Figure 5

RTG-2 Cells stained with Hematoxylin-Eosin

RTG-2 cells were grown in Leighton tubes until confluent, the cells were then fixed with methanol and stained with hematoxylin-eosin.
(X 250)

Figure 6

RTG-2 Cells infected with VR-299 IPN Virus and stained with Hematoxylin-Eosin

Confluent monolayer of RTG-2 cells in Leighton tubes were infected with 0.1 ml of 1/1000 dilution of stock virus and incubated at 18°C. At 3 days after infection, cpe was evident and cells were then fixed with methanol and stained with hematoxylin-eosin. Cytoplasmic shrinkage resulting in strandy appearance was the predominant feature in infected cells.
(X 250)

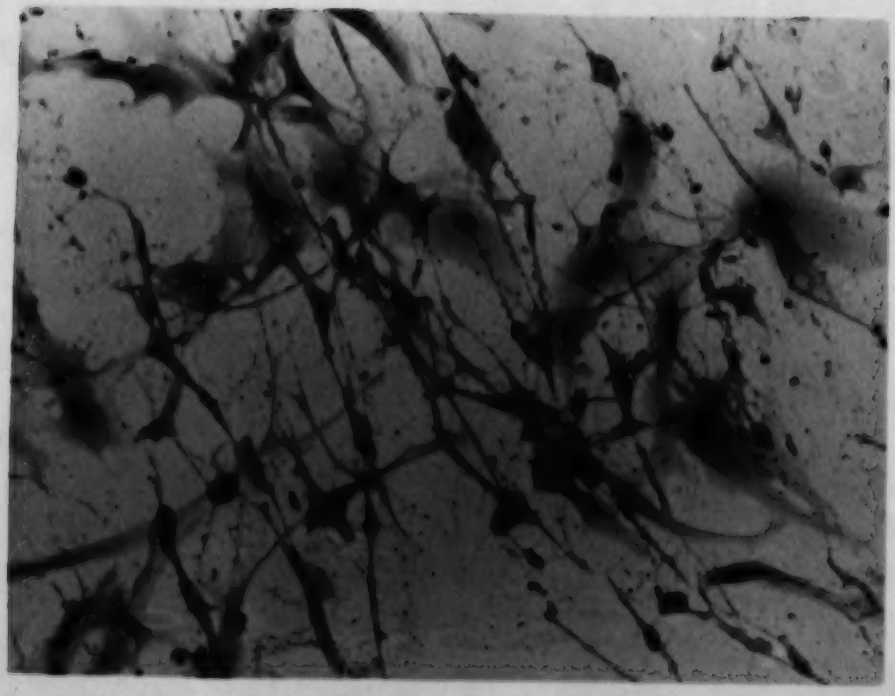


Figure 7

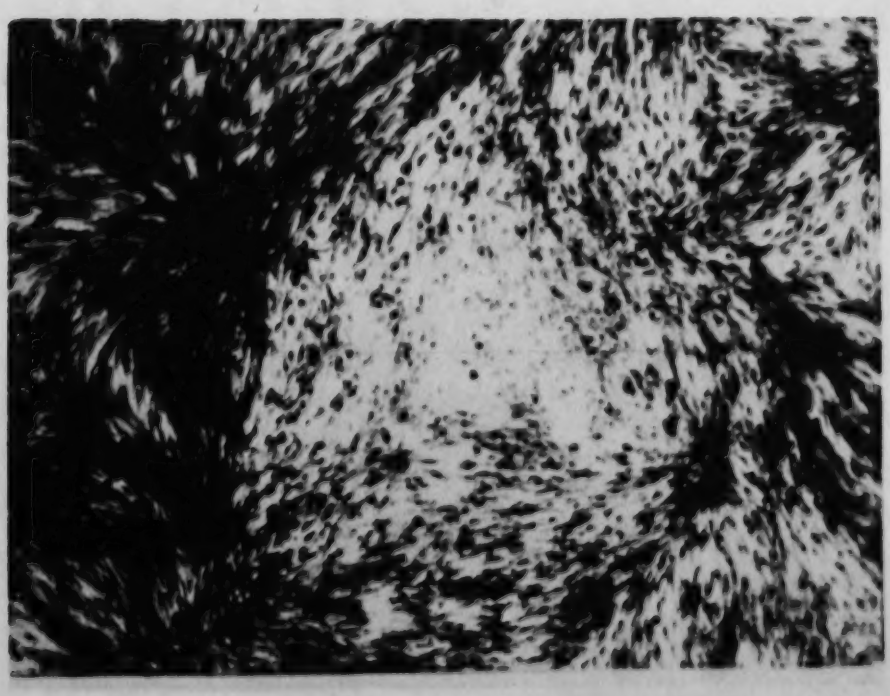
RTG-2 Cells stained with Crystal Violet

RTG-2 cells were grown in 60 mm tissue culture dishes until confluent. Cells were then overlayed with MEM overlay medium as described in materials and methods and incubated at 18°C for 3 days. Cells were then fixed with 30% formaldehyde and stained with crystal violet. The fibroblastic morphology is characteristic of normal RTG-2 cells.
(X 150)

Figure 8

Plaque formed by infection of RTG-2 Cells with VR-299 IPN Virus

Confluent RTG-2 cells in 60 mm tissue culture dishes were infected with 0.1 ml of 1/1000 dilution of stock virus and overlayed with MEM overlay medium as described in materials and methods. At 3 days after infection the cells were fixed with 30% formaldehyde and stained with crystal violet.
(X 150)



2. Virus Dose-Response Curves of IPN Virus in CHSE-214 and RTG-2 Cells

Virus dose-responses were performed to determine (a) the reliability of using CHSE-214 cells for titration of virus infectivity by the plaque assay method, (b) the effect of using a single and double overlay medium on the plaquing efficiency of IPN virus in CHSE-214 cells, (c) the plaquing efficiency of IPN virus in CHSE-214 and RTG-2 cells and (d) the effect of using 1 X and 0.5 X MEM on the plaquing efficiency of IPN virus in CHSE-214 cells. Virus dose-responses were performed in the following manner: two-fold dilutions of a virus suspension in Tris-HCL buffer were prepared. One tenth ml of the virus suspension was then added into 60 mm tissue culture dishes containing confluent cultures of either CHSE-214 cells or RTG-2 cells in duplicate. Plaques were determined as described in materials and methods; 2 X MEM (containing 2 X MEM, 0.16% sodium bicarbonate, 28 mM HEPES, 20% FCS, 200 IU/ml of penicillin and 200 µg/ml of streptomycin) was also employed in comparative experiments. Medium that contained 1 X MEM, 0.08% sodium bicarbonate, 14 mM HEPES, 20% FCS, 100 IU/ml of penicillin and 100 µg/ml of streptomycin will be referred to simply as 1 X MEM.

(a) Reliability of plaque formation in CHSE-214 cells

A virus dose-response was performed with two-fold dilutions of a virus suspension containing approximately 8.5 X 10^2 PFU/ml of VR-299 IPN virus. The result in Fig 9 revealed a linear relationship between the dilution of virus inoculated and the number of plaques that developed. Titration of log dilutions also revealed the same result (Fig 10). Thus, under the conditions employed, CHSE-214 cells was shown to be useful for

Figure 9

Linear Dose-Response of VR-299 IPN Virus in CHSE-214 Cells

Virus dose-response was performed with two-fold dilutions of a virus suspension containing approximately 8.5×10^2 PFU/ml of VR-299 IPN virus. Confluent CHSE-214 cells in 60 mm tissue culture dishes were infected with 0.1 ml of the diluted virus. After adsorption, infected cell cultures were then overlayed with 2 X MEM plus Agarose (0.5% final) and plaques were enumerated as described in materials and methods.

Symbols: O mean of titres from 4 samples.

I range of titres.

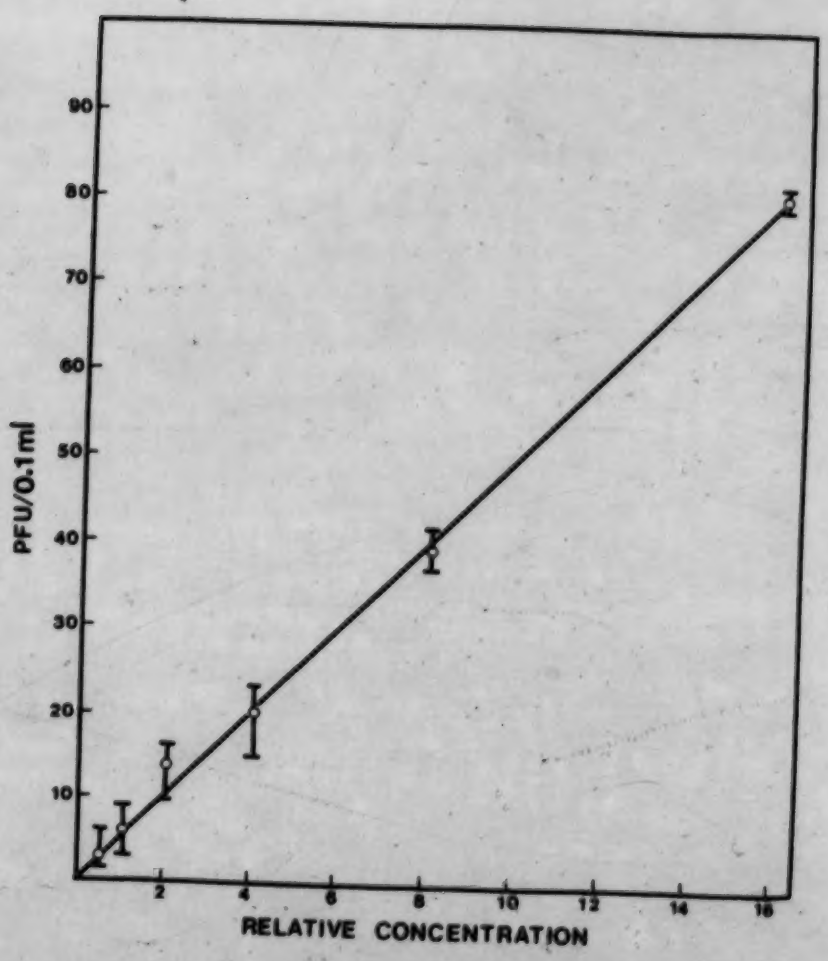
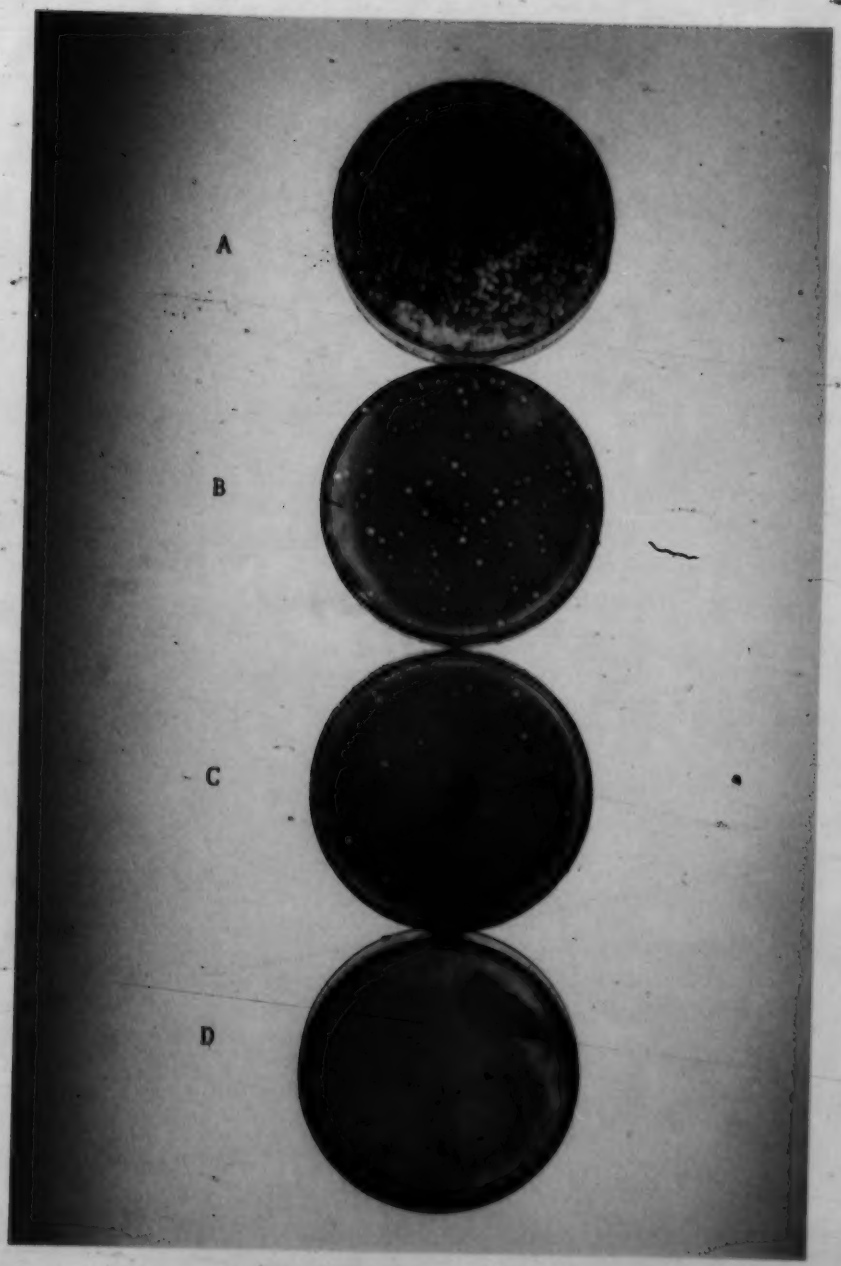

PFU/0.1ml
90
80
70
60
50
40
30
20
10
2
4
6
8
10
12
14
16
RELATIVE CONCENTRATION

Figure 10

Culture Dishes containing Plaques formed by IPN Virus in CHSE-214 Cells

Plaque assay of a virus suspension containing VR-299 IPN virus was performed as described in materials and methods.

A. Undiluted
B. 10^{-1} dilution
C. 10^{-2} dilution
D. Uninfected


A
B
C
D

the titration of virus infectivity.

(b) Effect of a single and double overlay medium on plaquing efficiency

With the plaque assay method described by Wolf and Quimby (1973), a second overlay fluid medium containing either HEPES or Tris as buffer is added to the first MEM:Agarose overlay. To determine the effect of the addition of a second overlay fluid medium on the plaquing efficiency of IPN virus-infected CHSE-214 cells, virus dose-response curves were determined with two sets of culture dishes containing confluent CHSE-214 cells. Each set was infected with two-fold dilutions of a virus suspension containing approximately 7 X 10^2 PFU/ml of VR-299 IPN virus. After adsorption of virus, both sets of culture dishes were overlayed with 6 ml of a 1:1 mixture of 2 X MEM and Agarose (1%). Four ml of 1 X MEM overlay medium was added to only one set of culture dishes. Plaques were then enumerated as described in materials and methods. The result in Fig 11 shows that no significant difference in plaquing efficiency was evident when either the single or double overlay medium was employed to titrate virus infectivity.

(c) Comparison of plaquing efficiency in CHSE-214 and RTG-2 cells

To determine the effect of using a single overlay medium of MEM:Agarose on the plaquing efficiency of virus-infected CHSE-214 and RTG-2 cells, virus dose-response curves for both cell lines were determined simultaneously with two-fold dilutions of a virus suspension containing approximately 7 X 10^2

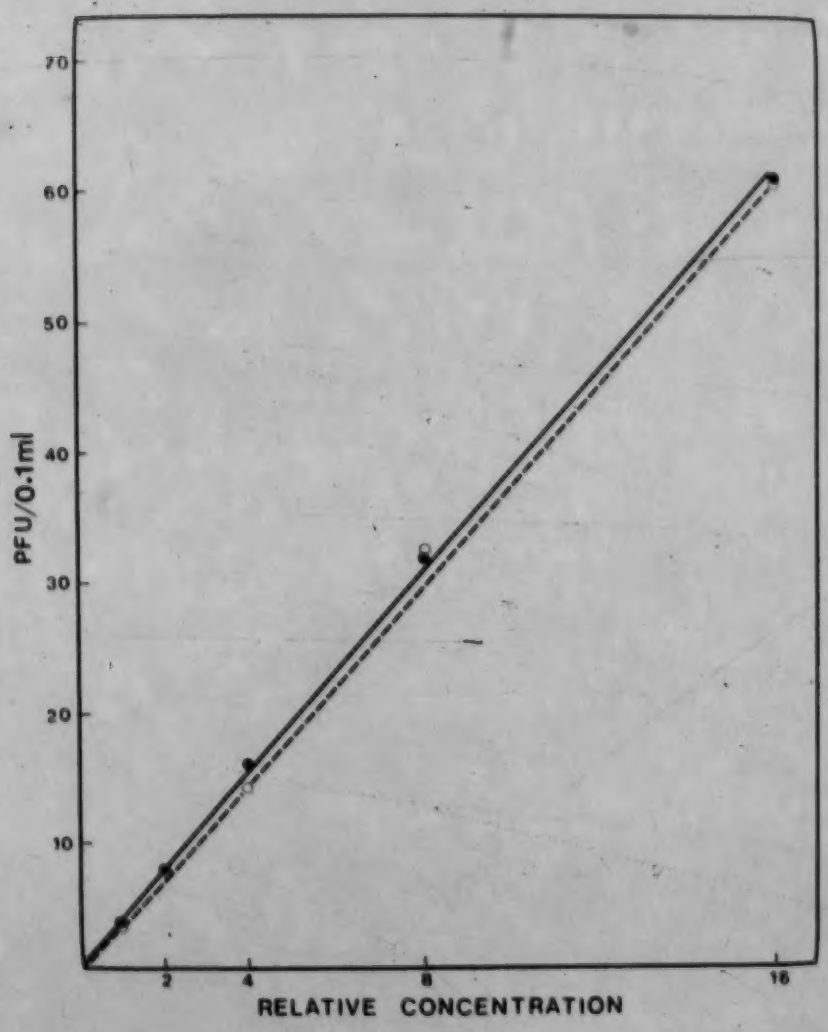
Figure 11

Linear Dose-Response Curves of VR-299 IPN Virus in CHSE-214 Cells using a Single and and Double Overlay Medium

Confluent cultures of CHSE-214 cells were inoculated with two-fold dilutions of a virus suspension containing approximately 7 X 10^2 PFU/ml of VR-299 IPN virus. After 1 h adsorption, culture dishes were overlayed with either a single overlay containing 2 X MEM plus Agarose (solid overlay) or a double overlay consisting of 2 X MEM:Agarose (solid overlay) and 1 X MEM (fluid overlay). Plaques were enumerated as described in materials and methods. Titres were obtained from an average of 2 samples. Since the values from two samples were similar, the range of titres is not given.

Symbols: ● Culture cells overlayed with a single overlay.

○ Culture cells overlayed with a double overlay.


70
60
50
40
30
20
10
PFU/0.1ml
2
4
8
16
RELATIVE CONCENTRATION

PFU/ml of VR-299 IPN virus. The result in Fig 12 shows that plaquing efficiency was significantly reduced in RTG-2 cells when compared to virus-infected CHSE-214 cells. The reason for the reduction in the number of plaques is not apparent. When a second overlay medium of 1 X MEM was added to the first overlay of MEM:Agarose in virus-infected RTG-2 cells but not to virus-infected CHSE-214 cells, the results in Fig 13 show that no significant difference in the number of plaques was observed between CHSE-214 and RTG-2 cells. These results show that an additional overlay fluid medium is not required for efficient plaque formation in CHSE-214 cells, while on the other hand, the double overlay was found to work best for plaque formation in RTG-2 cells.

(d) Effect of using 1 X and 0.5 X MEM on plaquing efficiency

To determine the effect of using 1 X and 0.5 X on the plaquing efficiency of virus-infected CHSE-214 cells, virus dose-responses were performed with two-fold dilutions of a virus suspension containing approximately 1.5 X 10^3 PFU/ml of VR-299 IPN virus. Each dilution was added to two sets of culture dishes of CHSE-214 cells. One set was overlayed with a mixture of 1 X MEM and Agarose (1%), while the remaining set was overlayed with a 1:1 mixture of 2 X MEM and Agarose (1%). Plaques were enumerated as described in materials and methods. The result in Fig 14 show that no significant difference in plaquing efficiency was evident when either 1 X or 0.5 X MEM was used. Thus, the reduction in the amount of salts and nutrients did not affect

the plaquing efficiency of IPN virus-infected CHSE-214 cells. As a consequence of this finding, the recipe as described in materials and methods for the plaque assay was employed throughout the rest of this study.

Figure 12

Linear Dose-Response Curves of VR-299 IPN Virus in CHSE-214 and RTG-2 Cells

Confluent culturs of CHSE-214 and RTG-2 cells were infected with two-fold dilutions of a virus suspension containing approximately 7 X 10^2 PFU/ml of VR-299 IPN virus. After 1 h adsorption, culture dishes were overlayed with a single overlay medium consisting of 2 X MEM and Agarose (0.5% final). Plaques were enumerated as described in materials and methods.

Symbols:

● Plaques formed in CHSE-214 cells. (Average of 4 samples)

○ Plaques formed in RTG-2 cells. (Average of 4 samples)

I Range of titres.

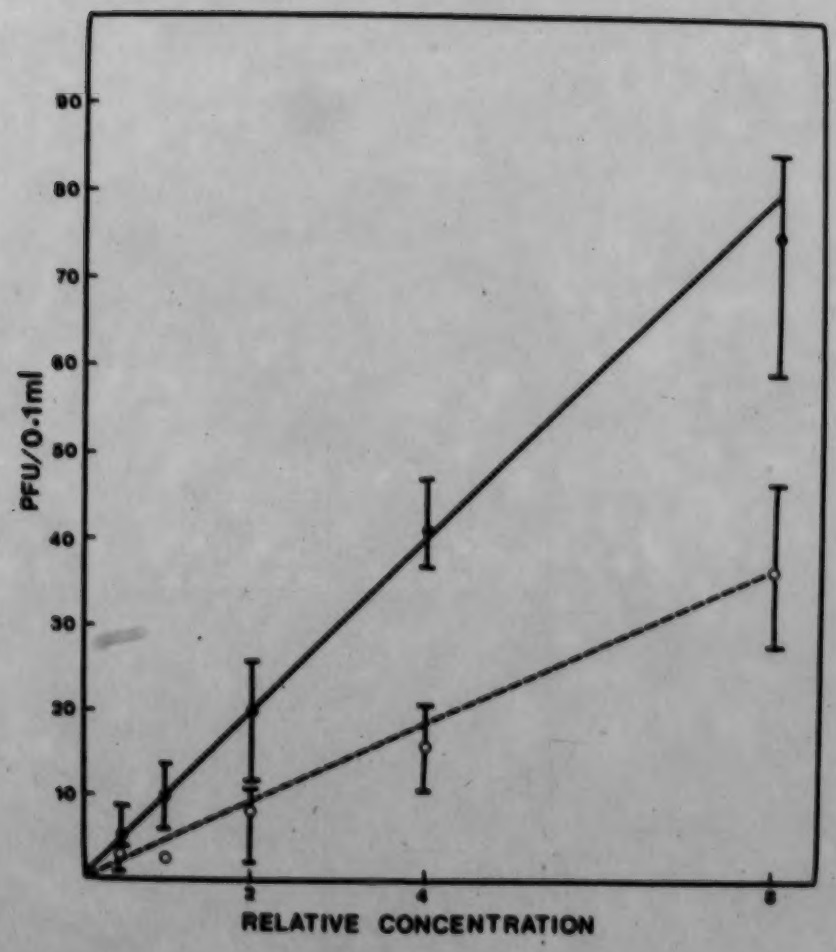

PFU/0.1ml
90
80
70
60
50
40
30
20
10
2
4
8
RELATIVE CONCENTRATION

Figure 13

Linear Dose-Response Curves of VR-299 IPN Virus in CHSE-214 and RTG-2 Cells

Confluent cultures of CHSE-214 and RTG-2 cells were infected with VR-299 IPN virus as described in fig 12 with the exception that an additional fluid overlay of 1 X MEM was added to RTG-2 cells. PFU values were an average of two samples.

Symbols:

- O Plaques formed in CHSE-214 cells overlayed with a single overlay medium.
- ● Plaques formed in RTG-2 cells overlayed with a double overlay medium.

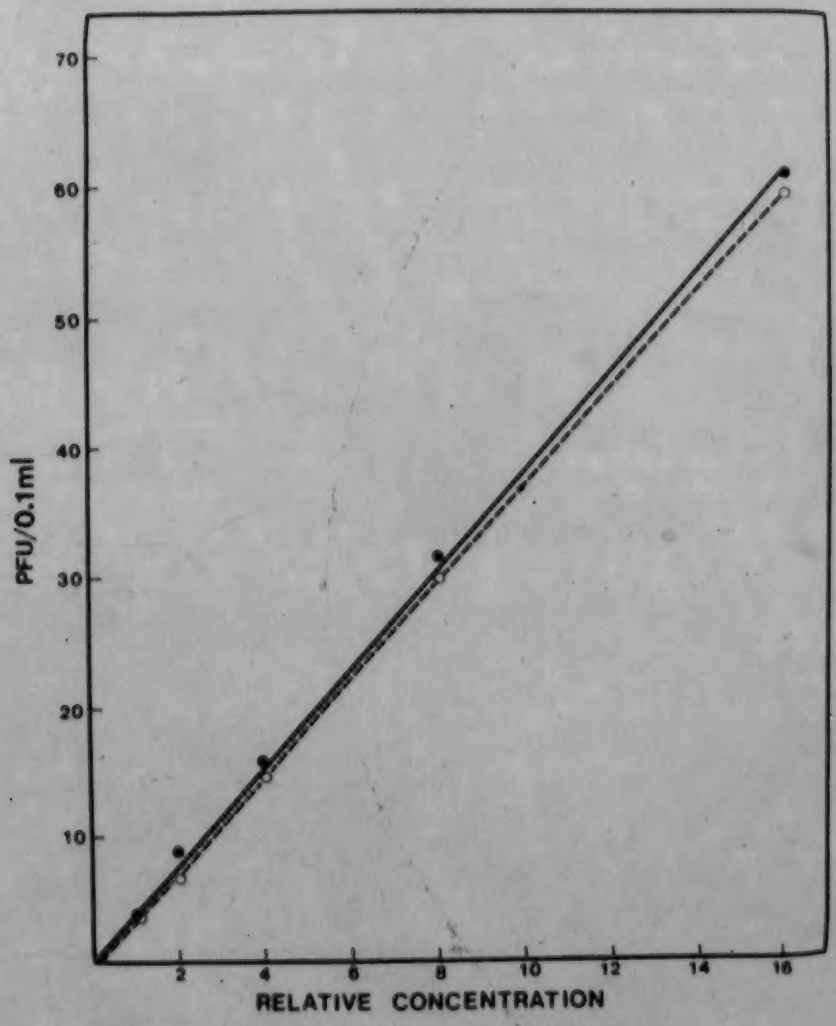

70
60
50
40
30
20
10
PFU/0.1ml
2
4
6
8
10
12
14
16
RELATIVE CONCENTRATION

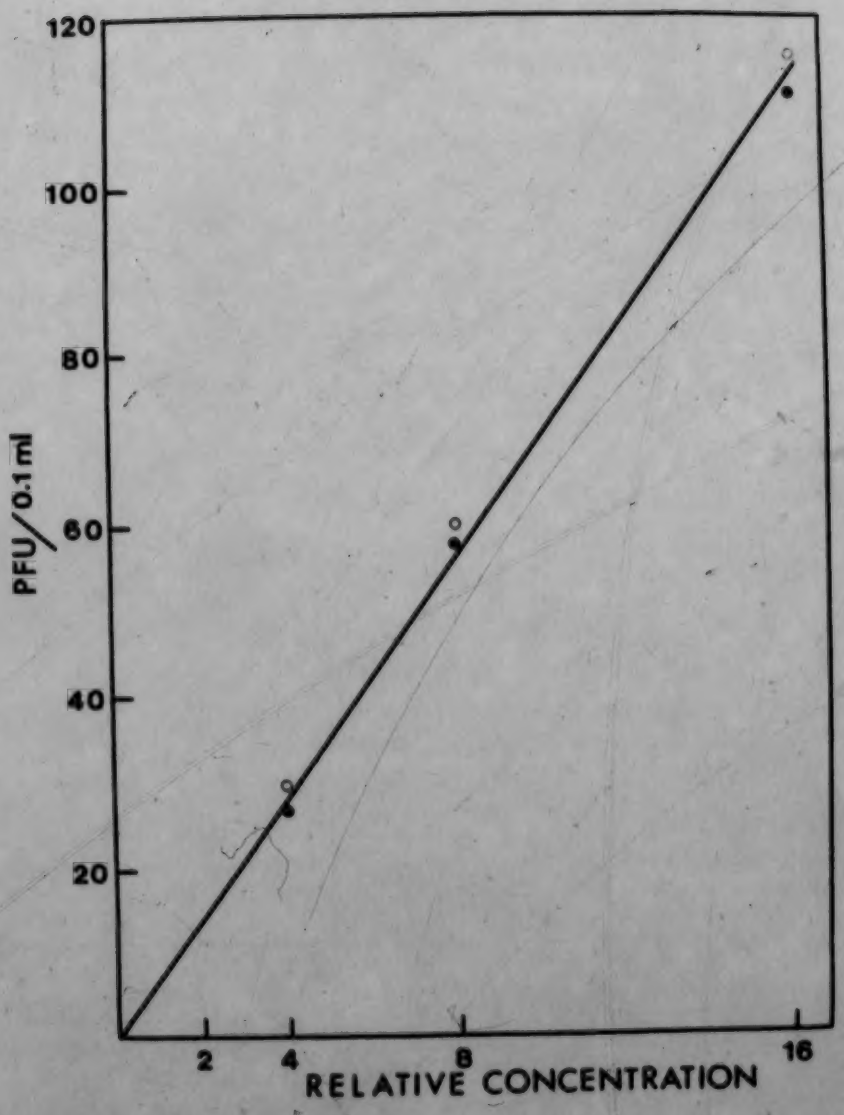
Figure 14

Effect of 0.5 X and 1 X MEM on the Plaquing Efficiency of VR-299 IPN Virus in CHSE-214 Cells

Confluent cultures of CHSE-214 cells were infected with two-fold dilutions of a virus suspension containing approximately 1.5 X 10^3 PFU/ml of VR-299 IPN virus. Infected cells were overlayed with either a 1:1 mixture of 1 X MEM and Agarose (1%) or a 1:1 mixture of 2 X MEM and Agarose (1%). Plaques were enumerated as described in materials and methods. PFU values were an average of 2 samples.

Symbols:

- O Culture cells overlayed with a single overlay medium containing 1 X MEM and 0.5% Agarose.
- ● Cultute cells overlayed with a single overlay medium containing 0.5 X MEM and 0.5% Agarose.


120
100
80
60
40
20
PFU/0.1 ml
2
4
8
16
RELATIVE CONCENTRATION

3. Adsorption of VR-299 IPN Virus to CHSE-214 Cells

The quantitative relationship between adsorption time and the number of plaques that developed was determined in the following manner: 0.1 ml of a virus suspension that contained approximately 1.3×10^3 PFU/ml of VR-299 IPN virus was added to confluent cultures of CHSE-214 cells after removal of culture medium. Adsorption was allowed to proceed up to 1 h at room temperature. At various intervals of time after virus inoculation, duplicate cultures were thoroughly rinsed three times with 5 ml of 1 X HBSS to remove unadsorbed virus. The culture dishes were then overlayed with MEM:Agarose and plaques were enumerated as described in materials and methods. The result in Fig 15 show that at 23°C, maximal adsorption occurred with 40 min of incubation, after which time there was no further increase in titre.

4. Stability of IPN Virus at Low Temperatures

(a) Stability of VR-299 and Jasper IPN virus at 4°C

Culture media from IPN virus-infected CHSE-214 cells were stored at 4°C, pH 7.3. The initial titre of VR-299 IPN virus in the culture medium was 2×10^8 PFU/ml, while the titre for the Jasper isolate was 1.8×10^7 PFU/ml. At the selected intervals of time, aliquots were withdrawn and assayed for virus infectivity. The result in Fig 16 shows that the Jasper isolate was more readily inactivated than the VR-299 isolate. A log reduction in titre occurred with storage for 50 days for the Jasper isolate and 90 days for the VR-299 isolate.

(b) Stability of VR-299 and Jasper IPN virus at -20°C and -65°C

Culture media from IPN virus-infected CHSE-214 cells

Figure 15

Adsorption of VR-299 IPN Virus to CHSE-214 Cells

One tenth of a ml of virus suspension containing approximately 1.3 X 10^3 PFU/ml of VR-299 IPN virus was added into confluent cultures of CHSE-214 cells. At selected times after infection, duplicate culture dishes were rinsed three times with HBSS to remove unadsorbed virus and plaque assays were performed as described in materials and methods.

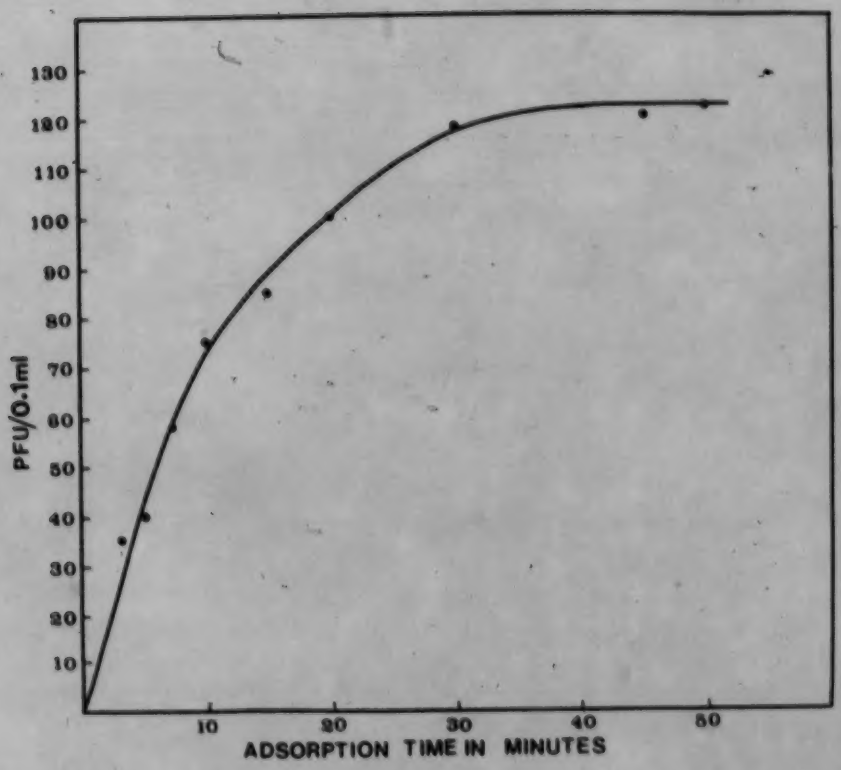

130
120
110
100
90
80
70
60
50
40
30
20
10
PFU/0.1ml
10
20
30
40
50
ADSORPTION TIME IN MINUTES

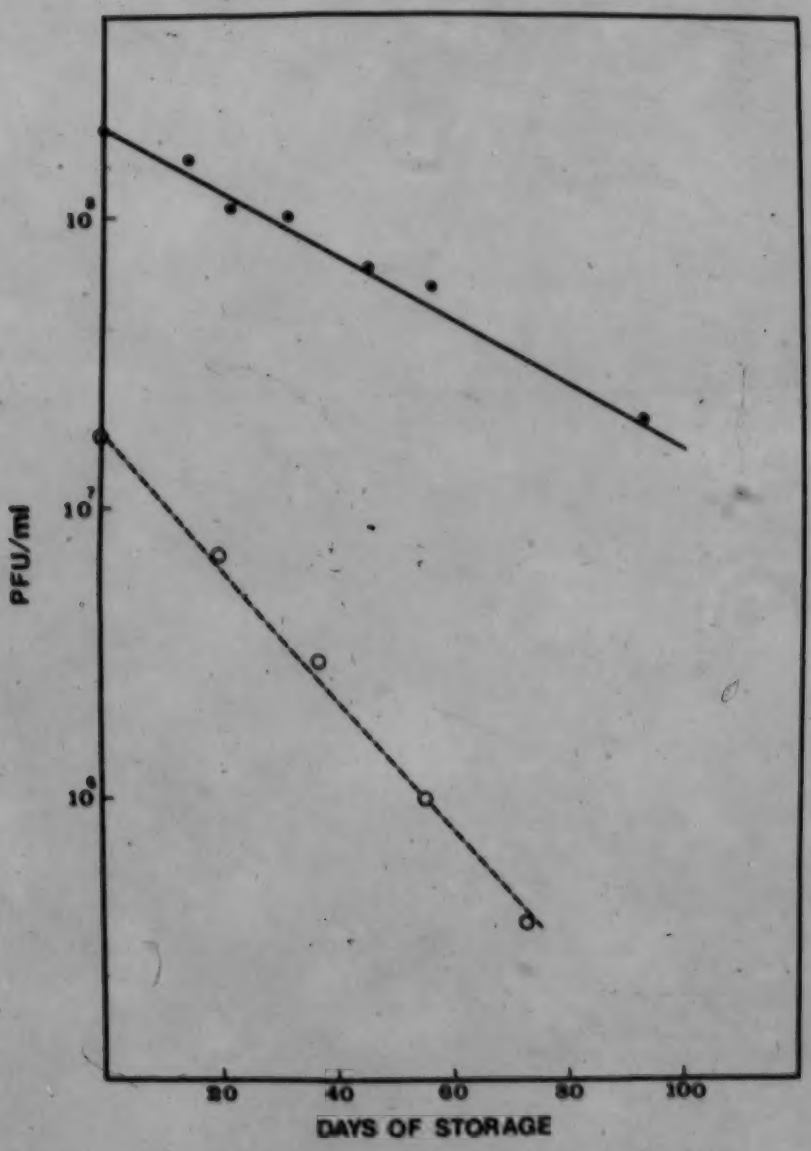
Figure 16

Stability of VR-299 and Jasper IPN Virus at 4°C

Culture media from IPN virus-infected CHSE-214 cells were stored at 4°C and aliquots were withdrawn at selected intervals of time and assayed for infectivity by the plaque assay method.

Symbols: ● VR-299 IPN virus

○ Jasper IPN virus


PFU/ml
10⁸
10⁷
10⁶
20
40
60
80
100
DAYS OF STORAGE

containing approximately 7 X 10^8 PFU/ml of VR-299 or Jasper IPN virus were stored in small aliquots (1 ml) at -20°C and -65°C. An aliquot was thawed and assayed for virus infectivity at selected intervals of time. The result in Table 1 shows at -20°C, 17% of the infectivity could be recovered for VR-299 and 24% for the Jasper isolate after one week storage, while at -65°C, 28% and 21% of the infectivity was recovered for VR-299 and Jasper respectively. Both virus isolates showed a dramatic loss of infectivity at -20°C after 5 months storage, while at -65°C, survival of both isolates was much greater and titres of 10^6 PFU/ml could be recovered. Thus, both virus isolates were more stable at -65°C and 4°C than at -20°C when stored for a long period of time.

5. Effect of pH on the Stability of VR-299 IPN Virus

The stability of VR-299 IPN virus suspended in buffers ranging from pH 3 to 11 were determined in the following manner: culture medium containing 5 X 10^6 PFU/ml of VR-299 virus from infected CHSE-214 cells was used as the starting material. One tenth of a ml of virus medium was added into 0.9 ml of the following buffer solutions prepared according to the procedures of McKenzie (1969): pH 3.0 (0.05 M Glycine-HCL); pH 4 and 5 (0.1 M acetic acid-sodium acetate); pH 6, 7 and 8 (0.1 M Na_2HPO_4-NaH_2PO_4); pH 9.0, 9.5, 10.0, 10.5, 10.8 and 11.0 (0.05 M Glycine-NaOH). The virus-buffer mixtures were incubated for 1 h at 23°C, after which time the mixtures were dialyzed against 0.1 M K_2HPO_4-KH_2PO_4 neutralizing buffer, pH 7.0 for 12 h. Log dilutions were then prepared with MEM growth medium, pH 7.4 and the $TCID_{50}$ end-points were determined as described in

Table 1

Stability of IPN Virus at Low Temperatures

Virus isolate	Storage	(PFU/ml)	
		-20°C	-65°C
*VR-299	1 h	5.6×10^8	6.0×10^8
	1 week	1.2×10^8	1.8×10^8
	5 months	1.0×10^2	1.4×10^6
**Jasper	1 h	7.3×10^8	6.3×10^8
	1 week	1.7×10^8	1.3×10^8
	5 months	2.4×10^3	9.0×10^6

* Titre at beginning of experiment at 4°C was 7.0×10^8 PFU/ml.

** Titre at beginning of experiment was 7.2×10^8 PFU/ml.

materials and methods. The results in Fig 17a and Fig 17b show that VR-299 IPN virus was stable at pH 3 to 10 but not at pH 10.5 and higher.

Figure 17a

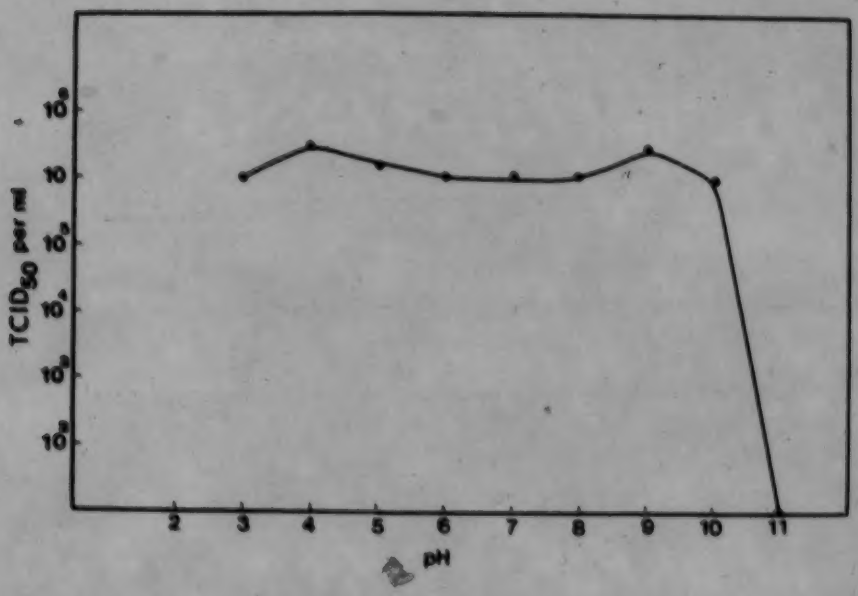
Effect of pH on the Stability of VR-299 IPN Virus

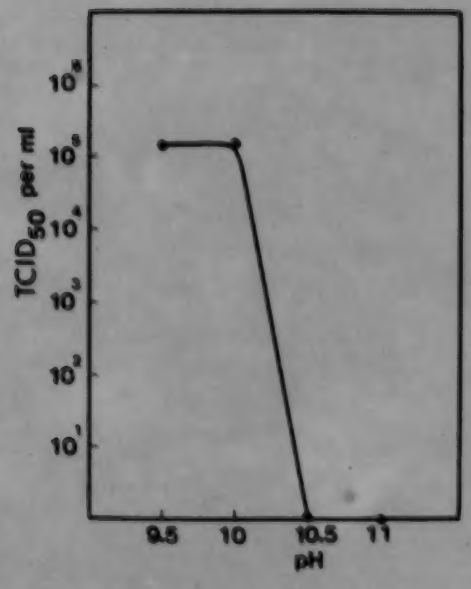
One tenth of a ml of virus suspension containing approximately 5 X 10^6 PFU/ml of VR-299 IPN virus was added into 0.9 ml of buffer solutions of pH 3 to 11 and incubated for 1 h at 23°C. At the end of this period the virus-buffer mixtures were neutralized by dialysis against neutralizing buffer as described in the text. Log dilutions were prepared and end-points were determined as described in materials and methods.

Figure 17b

Effect of pH on the Stability of VR-299 IPN Virus

The experiment was performed as described in fig 17a.


TCID50 per ml
pH
2
3
4
5
6
7
8
9
10
11


TCID50 per ml
pH
9.5
10
10.5
11

6. Growth Curve of IPN Virus in CHSE-214 Cells

The growth curve of VR-299 IPN virus and sequential development of cpe in CHSE-214 cells were determined by infecting confluent cultures of CHSE-214 cells in 3 oz prescription bottles with 0.4 ml of virus suspension at a multiplicity of infection of 37 PFU/cell. Virus adsorption was allowed to proceed for 1 h at room temperature at which time the cultures were rinsed three times with 5 ml of PBS to remove unadsorbed virus. Cultures then received 10 ml of MEM growth medium plus 5% FCS and incubation was allowed to proceed at 18°C. At selected intervals of time after infection, duplicate samples were withdrawn, pooled for cell-associated virus (CV) and released virus (RV). Samples for the determination of RV were obtained by removing the culture medium, which was then centrifuged for 10 min at 2000 rev/min in a clinical centrifuge to pellet the cells and debri. Infectious virus in the media was then assayed as described in materials and methods. The infected cells were washed three times with 5 ml of PBS and 10 ml of MEM growth medium containing 5% FCS was added to the culture and the cell monolayer was dislodged from the glass by scraping with a rubber policeman. The cell suspension was sonicated for 3 min to release the CAV. Virus infectivity was assayed as described in materials and methods.

The growth curve of VR-299 IPN virus is shown in Fig 18. Observations on the appearance of cpe were made at selected times when samples were withdrawn for infectivity assay. Changes in the appearance of cell sheet were recorded as 1+, 2+, 3+ and 4+ cpe. A 1+ cpe denotes the stage of infection when no gross morphological changes in the cell sheet can be detected, however the infected cells appear slightly granular and

Figure 18

Growth Curve of VR-299 IPN Virus in CHSE-214 Cells infected with a Multiplicity of Infection of 37

Confluent cultures of CHSE-214 cells in 3 oz prescription bottles (4×10^6 cells/bottle) were inoculated with 0.4 ml of a virus suspension containing approximately 1.5×10^8 PFU of VR-299 IPN virus. After 1 h adsorption, cell cultures were washed three times with PBS and 10 ml of MEM growth medium plus 5% FCS was then added to infected cultures and incubated at 18°C. At selected intervals of time cultures were withdrawn and assayed for cell-associated (CAV) and released virus (RV).

Symbols:

● Cell-associated virus

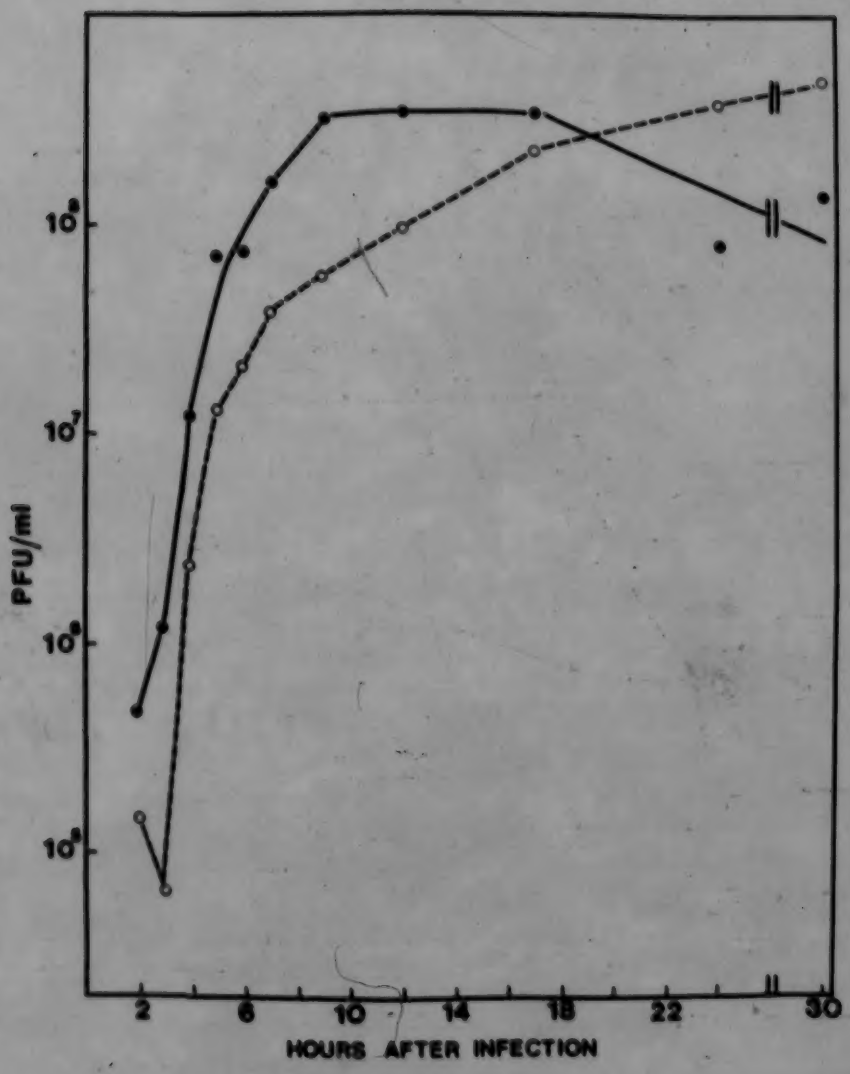
○ Released virus

\+ Infected cells appeared slightly granular and dense.

++ Infected cells began to round up, but cell sheet remained intact.

+++ cpe is reached in which 50% of cells have rounded up followed by pycnotic degeneration of cell sheet.

++++ cpe is reached in which 80% or more of cells have rounded up, with widespread detachment of cells from the glass surface.


PFU/ml
10^8
10^7
10^6
10^5
2
6
10
14
18
22
30
HOURS AFTER INFECTION

dense. A 2+ cpe was reached when cells began to round up, but the cell sheet remained intact. A 3+ cpe is reached when 50% of the cells have rounded up and pycnotic degeneration of the cell sheet became evident, as well as detachment of cells from the glass (although not widespread). A 4+ cpe is attained when 80% or more of cells have rounded up, the cell sheet no longer showed any form of confluency and spaces between groups of cells were prominent with widespread detachment of rounded cells from the glass surface. Clumping of cells also occurred throughout the glass surface and cellular debris suspended in the culture medium became clearly observable.

The growth curve in Fig 18 shows that an initial latent period of about 3 h was detected. This was followed by a rapid increase in intracellular virus (CAV). A maximum titre was reached by 10 h pi after which time the titre of infectious virus levelled off. Increase in RV followed closely after the increase in CAV. By 17 h pi, the number of virus recovered intracellularly decreased. This was followed by a corresponding increase in the recovery of RV up to 30 h pi. The titre of total detectable virus exceeded free or released virus. A 1+ cpe was first observed at 5 h pi, 2+ cpe by 7 h pi, 3+ cpe by 9 h and 4+ cpe by 12 h pi, at which time no further increase in intracellular virus was detected. The yield was 1.6×10^3 PFU per cell.

Two important observations were evident in the VR-299 IPN virus growth curve. Firstly, the appearance of cpe can be effectively used to monitor the rate of virus multiplication and production and secondly, approximately 35 to 45% of the infectious virus remained cell-associated at 30 h pi, and which will have to be considered when purification and

and concentration procedures are to be used. The results are in agreement with those of Cohen et al (1973) who reported that 60% or more of the infectious virus remained cell-associated at 48 h pi in RTG-2 cells. The actual percentage may vary from one preparation to another, depending on the time of harvest and the infectivity of the stock virus used for infecting the cells.

7. Growth Curve of Jasper IPN Virus

A one-step growth curve of Jasper IPN virus was determined in the same manner as for VR-299 IPN virus except that a multiplicity of infection of 1 PFU per cell was used. The result in Fig 19 show that an initial latent period of about 2 h was observed, followed by an exponential increase in CAV up to 10 h pi and reaching a maximum titre by 24 h pi, at which time the experiment was terminated. The increase in RV lagged slightly behind the increase in CAV, but maximum RV was attained at 24 h pi. The appearance of cpe was slow compared to the appearance of cpe in VR-299 infected cells. A 1+ cpe was detected at 10 h pi and at 24 h pi, at which time the experiment was terminated, the cpe was between 2+ and 3+. The experiment was not carried on long enough for the determination of the percentage of the virus that remained cell-associated.

Figure 19

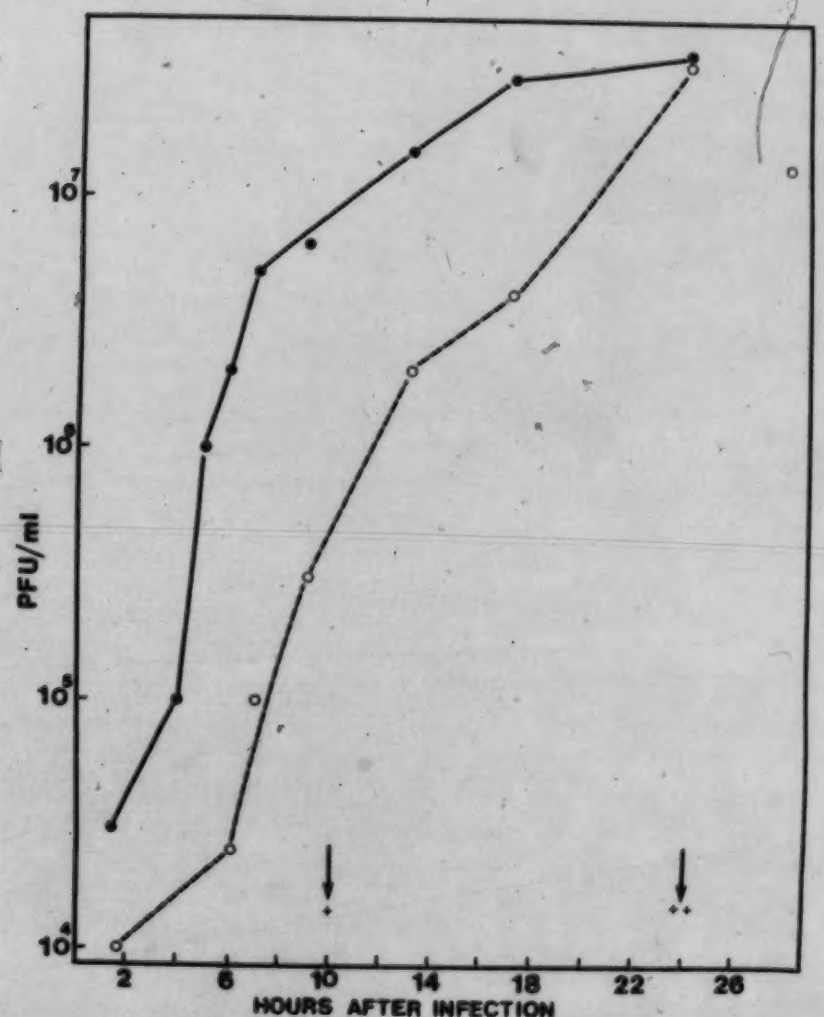
Growth Curve of Jasper IPN Virus in CHSE-214 Cells infected with One PFU per Cell

Confluent cultures of CHSE-214 cells in 3 oz prescription bottles (containing 4 X 10^6 cells per bottle) were infected with 0.4 ml of a virus suspension containing approximately 4 X 10^6 PFU of Jasper IPN virus. The same procedure as described in fig 18 was followed.

Symbols: ● Cell-associated virus (CAV).

○ Released virus (RV).

\+
cpe as described in fig 18.
++


10^7
10^6
10^5
10^4
PFU/ml
2
6
10
14
18
22
26
HOURS AFTER INFECTION

8. Purification, Concentration and Recovery of Infectious IPN Virus

Confluent cultures of CHSE-214 cells were infected with IPN virus at low multiplicity of infection. For this purpose, a 1/1000 dilution of a fresh stock of virus suspension containing approximately 2.5×10^8 PFU/ml was used. At 3 days pi, a 4+ cpe was normally evident. Cultures were then harvested as described in materials and methods. A titre of approximately 2.5×10^8 PFU/ml of culture medium was normally recovered. At each stage in the purification procedure, a small aliquot was withdrawn and assayed for infectivity by the plaque assay method. All titrations were performed simultaneously.

(a) Concentration and recovery of infectious IPN virus by centrifugation

In this experiment, the cell-fluid mixture obtained from IPN virus-infected CHSE-214 cells was sonicated for 1.5 min and then centrifuged for 15 min at 6,000 rev/min in a Sorvall RC2-B centrifuge to remove cellular debris. The clarified supernatant containing the virus was further centrifuged for 2 h at 30,000 rev/min in a Beckman Model L-2 ultracentrifuge using the fixed angle Type 30 rotor. The supernatant was withdrawn and the pellet material was resuspended in a small volume of Tris-HCL buffer, pH 7.2. RNase at 20 µg/ml was added, and the sample was incubated for 1 h at 37°C. It was then layered onto a 20 to 40% CsCl gradient and centrifuged for 16 h at 35,000 rev/min in a Beckman Model L2-65B ultracentrifuge using the SW 50.1 rotor. The virus band was withdrawn with a sterile needle and syringe. Aliquots withdrawn from the clarified supernatant,

the high speed supernatant, the resuspended pellet material and the virus band in CsCl were assayed for infectivity as described in materials and methods.

The result in Table 2 show that of the total titratable infectious virus, only 2.8% of the infectivity was recovered in the virus pellet. This would seem to indicate that sedimenting the virus by high speed centrifugation results in a dramatic loss of infectivity. Whether the low infectivity was due to viral aggregation is not known. Centrifugation in CsCl had no harmful effect on the infectivity of the virus (Table 3). It can be concluded that high speed centrifugation of medium containing the virus is not a satisfactory method for concentrating IPN virus.

(b) Extraction of virus from virus-cell mixture with Freon 113

To determine the extent of recovery of infectious virus from Freon-extracted virus-cell mixture, infected CHSE-214 cells were removed from the glass surface of a Roux bottle by scraping with a rubber policeman. The cells were then sedimented at 6,000 rev/min in a Sorvall RC2-B centrifuge using the GSA rotor for 15 min at 4°C. The supernatant was removed and the cell pellet resuspended in a small volume of Tris-HCl buffer. An aliquot was withdrawn for infectivity assay. The remaining cell resuspension was then partitioned into 2 equal volumes, one volume was sonicated for 1.5 min with a Bronwill Bionic Model III sonicator (Bronwill Scientific, Rochester), and the remaining volume was treated with an equal volume of Freon as des-

Table 2

Recovery of Infectious VR-299 IPN Virus by High Speed Centrifugation

Virus fraction	PFU/ml	Vol (ml)	Total PFU	%
Clarified low-speed supernatant	1.8×10^8	300	5.4×10^{10}	100
High speed supernatant	2.8×10^6	300	8.4×10^8	1.4
Resuspended virus pellet from high speed supernatant	1.9×10^8	8	1.5×10^9	2.8
Banded in CsCl	1.2×10^9	1	1.2×10^9	2.2

Table 3

Recovery of Infectious VR-299 IPN Virus after CsCl Centrifugation

Virus fraction	Vol (ml)	PFU/ml	Total PFU	%
Virus resuspended from the pellet (from 30,000 rev/min centrifugation)	4	2.8×10^7	1.1×10^8	100
Banded in CsCl	1	1.1×10^8	1.1×10^8	100

cribed in materials and methods. Aliquots were removed from both cell suspensions and assayed for infectivity.

The results are shown in Tables 4 and 5. It is evident that more infectious virus could be recovered by Freon extraction than by sonication. Thus, disruption of cells dose not necessary liberate all the virus from the cells. Much more rigorous sonication may be necessary to release all the virus, however, this would simply increase the risk of inactivating the virus. The results are in agreement with those of Cohen et al (1973). The reason for the tendency of the virus to adhere to cellular material is not known.

(c) Recovery of infectious IPN virus by CsCl gradient centrifugation

The small volume that is obtained by Freon extraction of a virus-cell suspension can easily be layered onto a CsCl gradient. Since the cellular debris is found in the interphase on treatment with Freon, the virus-containing aqueous phase is thus relatively free of much of the cellular material. The partially purified virus can be banded in a CsCl gradient in the absence of much of the cellular material. The result in Table 6 shows the excellent recovery of infectious VR-299 virus from a CsCl gradient. The virus band in the centrifuge tube is clearly evident (Fig 20). No analogous band is detected in the control tube that contained material from Freon-extracted uninfected CHSE-214 cells. That the band contained the virus is shown by electron microscopy (Fig 21) and infectivity assays. Most of the

Table 4

Recovery of Infectious Jasper IPN Virus from Cells treated with a Sonifier and Freon 113

Virus fraction	Vol (ml)	PFU/ml	Total PFU	%
Untreated virus-cell resuspension	2.0	5.0×10^8	1.0×10^9	100
Virus-cell sonicate	2.0	6.1×10^8	1.2×10^9	120
Extracted with Freon	2.0	6.5×10^8	1.3×10^9	130

Table 5

Recovery of Cell-associated VR-299 IPN Virus from Cells treated with a Sonifier and Freon 113

Virus fraction	Vol (ml)	PFU/ml	Total PFU	%
Sonicated virus-cell resuspension	5.0	1.5×10^9	7.5×10^9	100
Extracted with Freon	4.5	2.1×10^9	9.5×10^9	124

Table 6

Recovery of Freon-extracted VR-299 IPN Virus from a CsCl Gradient Centrifugation

Virus fraction	Vol (ml)	PFU/ml	Total PFU	%
Freon extract	4.0	1.5×10^9	6.0×10^9	100
Banded in CsCl	0.9	7.4×10^9	6.7×10^9	112

Figure 20

CsCl Gradient Centrifugation of Freon extracted and PEG concentrated VR-299 IPN Virus

Virus was purified and concentrated by CsCl gradient centrifugation at 35,000 rev/min for 16 h using the SW 50.1 rotor. The virus band is the lowest band near the bottom of the centrifuge tube shown in tubes A and D.

A. Freon extracted VR-299 IPN virus from CHSE-214 cells.

B. Freon extracted material from uninfected CHSE-214 cells.

C. PEG concentrated material from culture medium of uninfected CHSE-214 cells.

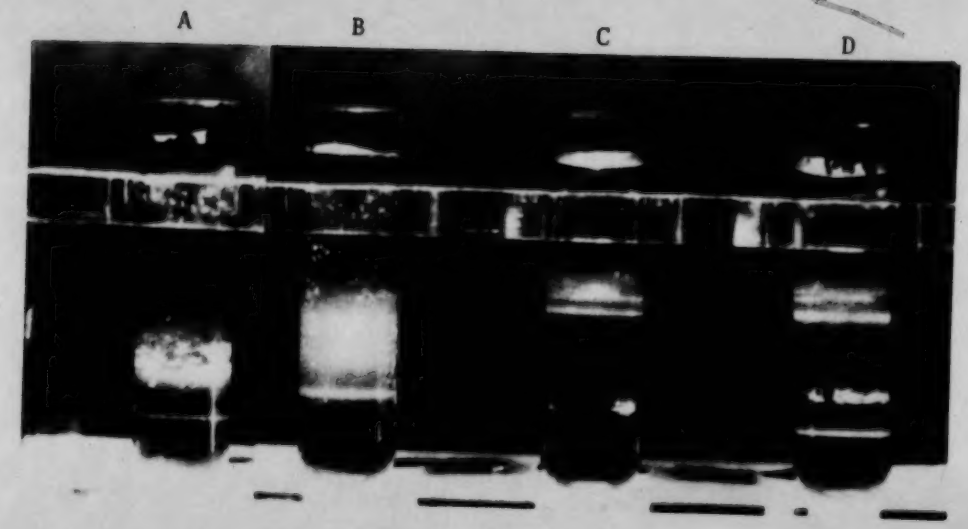
D. PEG concentrated VR-299 IPN virus from culture medium of infected CHSE-214 cells.

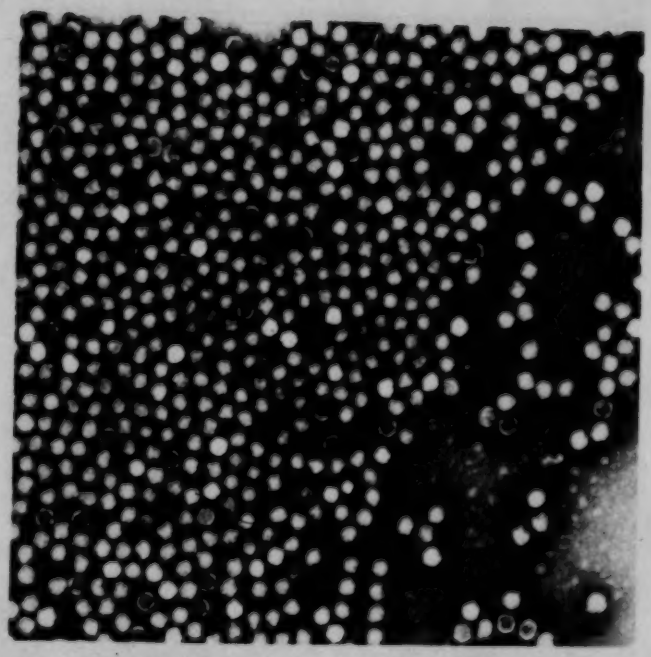
Figure 21

Electron Photomicrograph of VR-299 IPN Virus Negatively stained with PTA

Virus was purified as described in fig 20.

Magnification: 61,600 X
Bar: 100 nm


A
B
C
D



cellular debris remained at the top of the gradient. The same result is achieved with Jasper IPN virus (Fig 22).

(d) Concentration and recovery of IPN virus by PEG

To sediment virus to a pellet directly from a culture medium by high speed centrifugation (Kelly and Loh, 1972) is difficult because of the large volume of medium involved and the inactivation of a large percentage of the virus. Concentration of virus by PEG was therefore attempted. Culture medium containing virus was treated with PEG as described in materials and methods. Aliquots were withdrawn before and after PEG treatment, and after CsCl gradient centrifugation. The samples were then simultaneously assayed for virus infectivity.

The result in Table 7 show that PEG precipitation did not inactivate the virus. The recovery of infectious virus in the final concentrate was near 100%. About 1% of the total titratable virus remained in the culture supernatant after centrifugation at 10,000 rev/min (16,300g). The percentage of virus not sedimented was found to vary from one preparation to another. The overall loss of infectivity was approximately 4%, while 85% of infectious virus was recovered and concentrated by CsCl gradient centrifugation. The virus appeared as a sharp band in the centrifuge tube (Figures 20c, 20d and 22c). No such band was detected in the control tube that contained PEG-precipitated material from culture medium of uninfected CHSE-214 cells. It can be concluded that infectious virus in the culture medium could be concentrated 100 fold or more by precipitation

Figure 22

Purification and Concentration of Jasper IPN Virus by CsCl Density Gradient Centrifugation

Upper: Virus was centrifuged at 35,000 rev/min for 16 h at 4°C in a Beckman L2-65B ultracentrifuge using the SW 50.1 rotor.

A and B: Freon extracted virus from infected CHSE-214 cells. The virus band is the lowest band in the centrifuge tube.

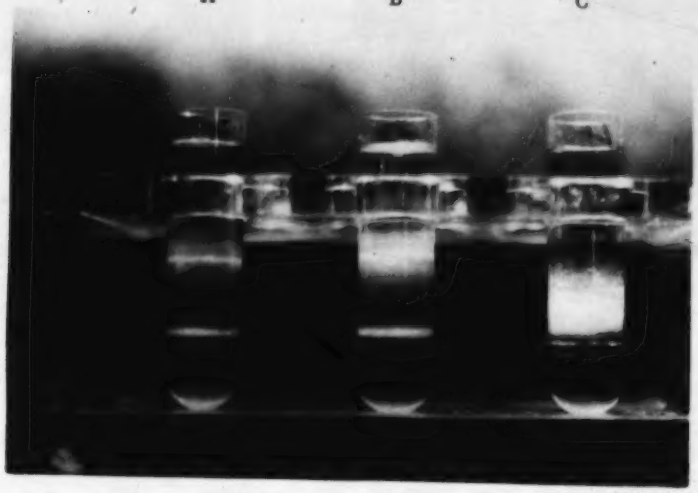
C: PEG concentrated virus from infected CHSE-214 cells. Culture supernatant from infected cells was treated with 5% PEG and 2.2% NaCl and precipitable material was sedimented as described in materials and methods.

Middle: The virus bands from A, B and C were withdrawn and pooled and further centrifuged in a CsCl gradient for 3 h at 4°C. The virus forms a sharp band near the bottom of the tube.

Lower: Electron photomicrograph of Jasper IPN virus from the second purification in CsCl and negatively stained with PTA.

Magnification: 41,000 X
Bar indicates 150 nm

A B C



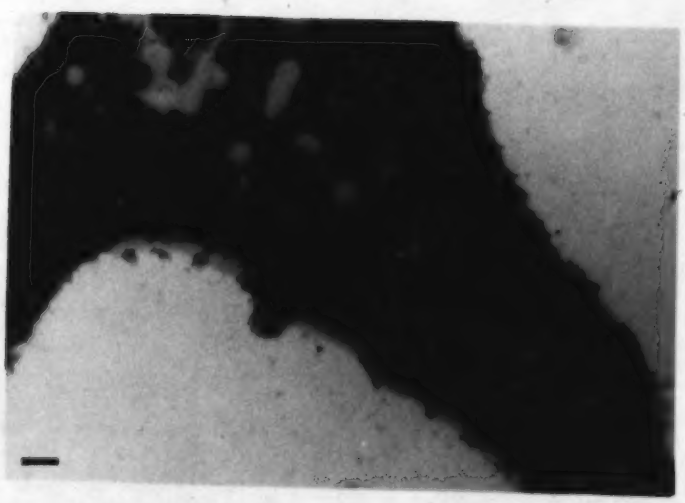


Table 7

Concentration and Recovery of Infectious VR-299 IPN Virus by PEG precipitation

Virus fraction	Vol (ml)	PFU/ml	Total PFU	%
Culture supernatant	464.0	1.35×10^{9}	6.26×10^{11}	100
Culture supernatant plus 5% PEG	485.0	1.29×10^{9}	6.30×10^{11}	100
PEG concentrate	5.0	1.25×10^{11}	6.25×10^{11}	99
Banded in CsCl*	3.5	1.50×10^{11}	5.30×10^{11}	85

* PEG concentrated virus was layered onto a 20 to 40% CsCl gradient and then centrifuged at 35,000 rev/min for 16 h at 4°C using the SW 50.1 rotor.

with 5% PEG with very little loss of infectivity. The PEG concentrated virus when resuspended in Tris-HCl buffer, pH 7.3 appeared very turbid and possibly also contained PEG that was carried over with the precipitable material. Addition of a few drops of 40% CsCl to the resuspension followed by low speed centrifugation in a clinical centrifuge was found to be adequate in removing any PEG present in the virus resuspension, since it will remain with the pellet which is discarded.

(e) Effect of CsCl gradient centrifugation on virus infectivity

It was observed that virus could be banded when centrifuged for 16 h at 35,000 rev/min (figures 20 and 22) in a CsCl gradient. Initial observations of SDS-polyacrylamide gel electrophoresis of IPN virus after a single centrifugation in CsCl at 35,000 rev/min (114,300g) for 16 h revealed numerous protein bands that were later shown to be absent in gels of IPN virus obtained from a second purification in CsCl. Since it has been found necessary to purify IPN virus at least twice in a CsCl gradient, studies were performed to determine the extent of inactivation of the virus under such conditions.

Freon-extracted and PEG concentrated IPN virus were pooled and layered onto a CsCl gradient as described in materials and methods and centrifuged for 16 h at 35,000 rev/min. The virus band was removed and a small aliquot was withdrawn for infectivity assay. The virus was further centrifuged in CsCl as before, but only for 6 h after which time an aliquot was with-

drawn for infectivity assay. The virus was recentrifuged for the third and final time for 6 h at 35,000 rev/min and the infectivity of the virus band was assayed.

The results in Table 8 show that a certain fraction of the virus was inactivated as the number of centrifugation in CsCl was increased. Of the total titratable virus, 80% of the infectivity could be recovered from the second purification in CsCl, while 57% was still recovered after the third centrifugation. The detrimental effect of several centrifugations was slightly more dramatic at centrifugal speed of 45,000 rev/min (189,000g). This is shown in Table 9. Only 17% of the infectivity was recovered after the third centrifugation. It is evident from these results that the number of infectious virus recovered decreases as the number of centrifugation and centrifugal speed increases. Under these conditions, the virus appeared to be unstable.Subsequent studies showed that virus could be banded by centrifugation for 3 h at 35,000 rev/min in the second purification step in CsCl. The 16 h centrifugation time in the first CsCl gradient centrifugation is required to separate the virus band from the cellular debris. The procedures as described in materials and methods was therefore employed throughout this study for the purification of all the IPN virus isolates.

The absorption spectrum (200-300 nm) of VR-299 and Jasper IPN virus obtained from the second purification in CsCl as described in materials and methods are shown in Fig 23 and Fig

24. The $A_{260}/_{280}$ ratio obtained from the spectrum was 1.25 for VR-299 IPN virus and 1.24 for the Jasper IPN virus isolate. The $A_{260}/_{280}$ ratios for the remaining 8 IPN virus isolates were found to vary from 1.20 to 1.25.

Table 8

Purification and Recovery of Infectious VR-299 IPN Virus by CsCl Gradient Centrifugation

Virus fraction	Vol (ml)	PFU/ml	Total PFU	%
Combined Freon extract and PEG concentrate	7.5	5.3×10^9	3.97×10^{10}	100
Banded once in CsCl*	2.8	1.5×10^{10}	3.90×10^{10}	98
Banded X 2 in CsCl**	2.0	1.6×10^{10}	3.20×10^{10}	80
Banded X 3 in CsCl**	1.5	1.6×10^{10}	2.25×10^{10}	57

* Virus was layered onto a 20 to 40% CsCl gradient and centrifuged for 16 h at 35,000 rev/min (114,300g) using the SW 50.1 rotor in a Beckman L2-65B ultracentrifuge at 4°C.

** Virus was layered onto a CsCl gradient and centrifuged for 6 h at 35,000 rev/min at 4°C in the L2-65B ultracentrifuge.

Table 9

Effect of High Speed CsCl Gradient Centrifugation on the Infectivity of VR-299 IPN Virus

Virus fraction	Vol (ml)	PFU/ml	Total PFU	%
Culture medium + 5% PEG	485	1.29×10^{8}	6.30×10^{11}	100
Banded once in CsCl*	3.5	1.50×10^{11}	5.30×10^{11}	85
Banded X 2 in CsCl**	3.5	9.50×10^{10}	3.30×10^{11}	53
Banded X 3 in CsCl**	1.5	7.10×10^{10}	1.10×10^{11}	17

* Virus was layered onto a 20 to 40% CsCl gradient and centrifuged for 16 h at 45,000 rev/min (189,000g) using the SW 50.1 rotor in a Beckman L2-65B ultracentrifuge.

** Virus was layered onto a 20 to 40% CsCl gradient and centrifuged for 6 h at 45,000 rev/min (189,000g) in the L2-65B ultracentrifuge.

Figure 23

Absorbance Spectrum of VR-299 IPN Virus

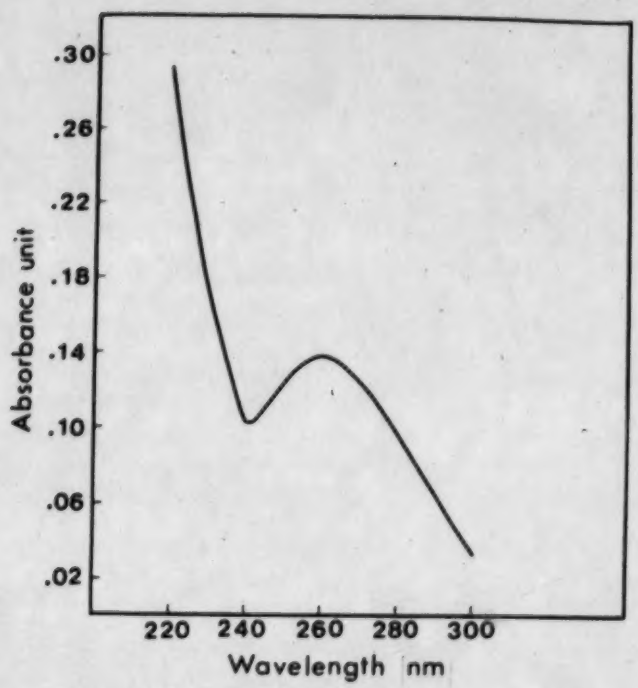
VR-299 IPN Virus was obtained from the second purification in CsCl as described in materials and methods. The absorbance spectrum was obtained with a Gilford 240 spectrophotometer.

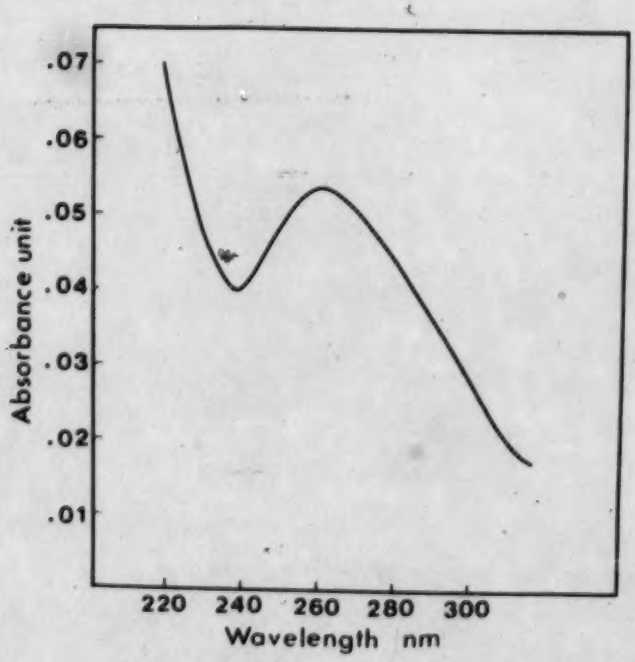
The $A_{260}/_{280}$ nm ratio of the virus is 1.25

Figure 24

Absorbance Spectrum of Jasper IPN Virus

Jasper IPN virus was obtained from the second purification in CsCl as described in materials and methods. The $A_{260}/_{280}$ nm ratio of the virus is 1.24


.30
.26
.22
.18
.14
.10
.06
.02
Absorbance unit
220
240
260
280
300
Wavelength nm


.07
.06
.05
.04
.03
.02
.01
Absorbance unit
220
240
260
280
300
Wavelength nm

9. Buoyant Density of IPN Virus in CsCl

The relationship between infectivity, absorbance at 260 nm and the buoyant density of VR-299 IPN virus was determined as described in materials and methods. The result in Fig 25 shows that purified virus from a 24 h centrifugation in CsCl has an A_{260} nm peak that corresponded to the fractions that contained the infectivity. The A_{260} nm absorbance values are however very low and may not be meaningful; the baseline values would seem to indicate otherwise. The buoyant density of the virus was found to be 1.33 g/cc.

The buoyant density of Jasper IPN virus was determined using radioactively labelled virions as described in materials and methods. As a control, the buoyant density of radioactively labelled VR-299 IPN virus was also determined at the same time. The results are shown in Fig 26 and Fig 27. The buoyant density of both virus isolates was found to be 1.33 g/cc in agreement with the value obtained by infectivity assay and A_{260} nm absorbance.

To determine the buoyant density in CsCl of FR, Bonnamy, d'Honnincthun, Reno, West Buxton, Powder Mill, Buhl and Western IPN virus isolates, purified isolates were centrifuged in individual tubes to equilibrium in CsCl for 24 h at 35,000 rev/min. As a control, VR-299 isolate was also centrifuged at the same time. From each tube containing the band of the appropriate virus isolate, an aliquot was withdrawn from the top, centre and bottom of the virus band with a capillary tube and the R.I. was determined as described in materials and methods. All IPN virus isolates were found to have a buoyant density of 1.33 g/cc.

The buoyant density value obtained for VR-299 IPN virus is in

agreement with the values reported for VR-299 IPN virus by Kelly and Loh (1972) and Dobos (1976), but not to that obtained by Cohen et al (1973).

Figure 25

Sedimentation of VR-299 IPN Virus in a CsCl Gradient

Virus purified from a second CsCl purification as described in materials and methods was layered onto a 20 to 40% CsCl gradient and centrifuged for 24 h at 35,000 rev/min at 4°C after which time fractions were collected and assayed for infectivity, determination of buoyant density and A_{260} nm absorbance.

Symbols: o- - -o Absorbance at A_{260} nm

●——● Infectivity

▲——▲ Buoyant density

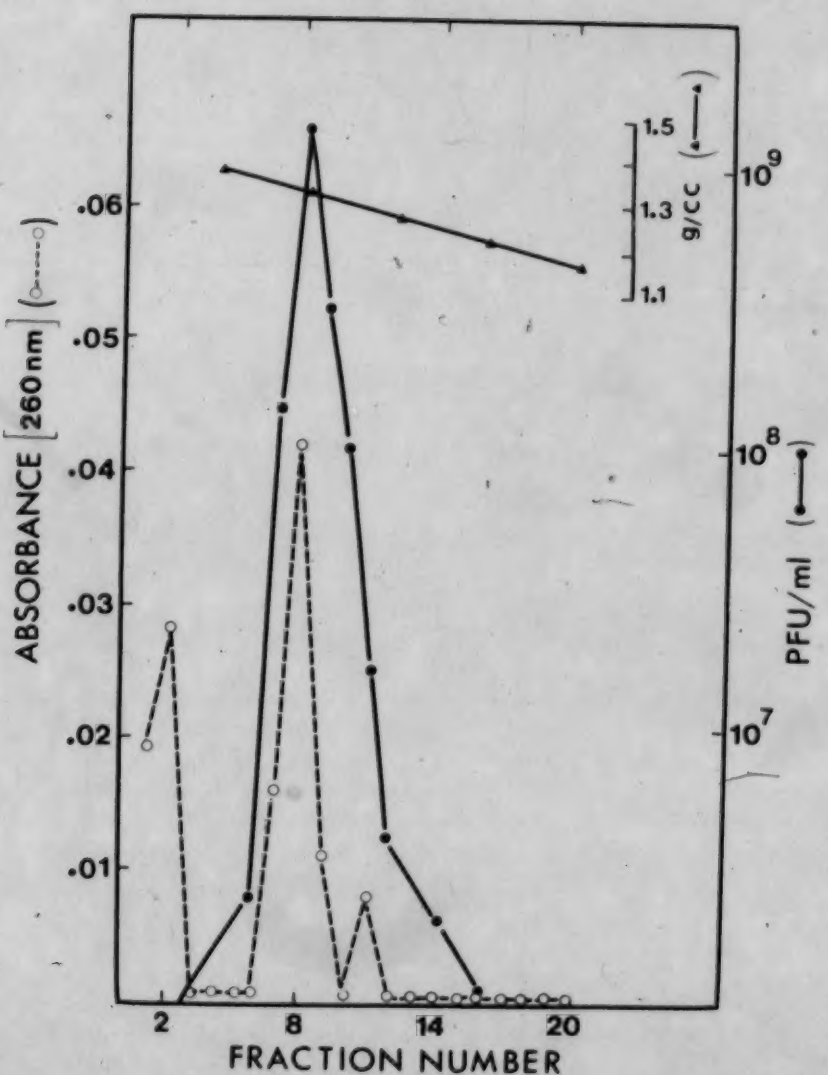

ABSORBANCE [260nm] (o----o)
.06
.05
.04
.03
.02
.01
1.5
1.3
1.1
g/cc (•—•)
10^9
10^8
10^7
PFU/ml (•—•)
2
8
14
20
FRACTION NUMBER

Figure 26

Isopycnic Sedimentation of purified VR-299 IPN Virus in a CsCl Gradient

Radioactively labelled virus was centrifuged for 24 h at 35,000 rev/min at 4°C after which time the buoyant density of the virus was determined as described in materials and methods.

Symbols: ● $[^3H]$-Guanine

□ Buoyant density

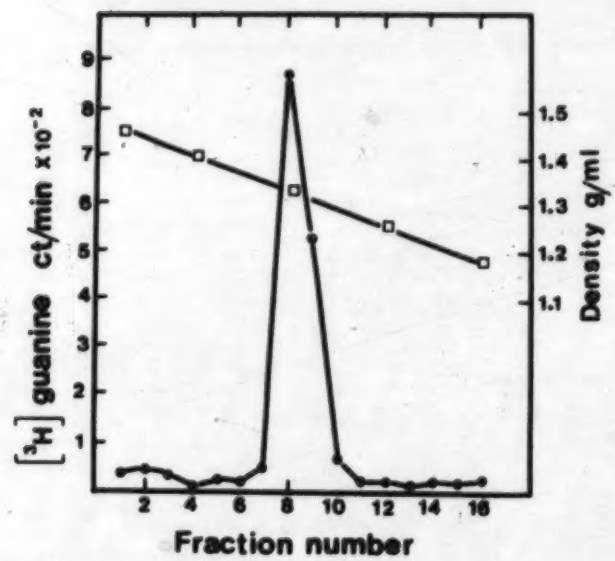
Figure 27

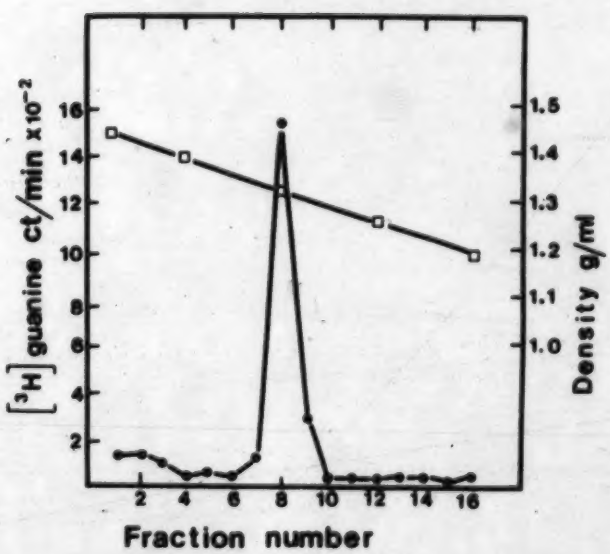
Isopycnic Sedimentation of purified Jasper IPN Virus in a CsCl Gradient

Radioactively labelled virus was centrifuged for 24 h at 35,000 rev/min at 4°C in a 20 to 40% CsCl gradient as described in materials and methods.

Symbols: ● $[^3H]$-Guanine

□ Buoyant density


[3H] guanine ct/min x 10-2
Density g/ml
Fraction number


[3H] guanine ct/min x 10-2
Density g/ml
Fraction number

10. Electron Microscopic Observations of IPN Virus

Electron microscopic observations were performed for all 10 IPN virus isolates purified from a second CsCl gradient centrifugation. Negative staining of virus was performed as described in materials and methods. It is evident from the results shown in Figures 28 to 37 that all the IPN virus isolates examined revealed the same morphological features in that they were isometric, hexagonal in profile and did not appear to possess the inner capsid structure that is characteristic of reovirus 2 (Fig 38). All IPN virus isolates were found to have a particle diameter of 74 nm, with distinct hexagonal symmetry consistent with the structure of an icosahedron (Caspar and Klug, 1962; Horne and Wildy, 1961). No breakdown of virus particles was evident although a few empty capsids were apparent in each preparation. The virus particles were homogeneous in size and particulate cellular material was noticeably absent in the virus preparation. In preparations that contained West Buxton and Reno virus isolates (Fig 32 and Fig 33 respectively), a few particles of smaller dimensions (58-60 nm) were evident. That they were detected in such small numbers (about 2% in the preparation containing Reno IPN virus) and their absence in the preparations of the other virus isolates indicated that they were probably not artifacts of the purification and centrifugation procedures. The relationship of these particles to infectious IPN virus particle is not known.

The results obtained for the size and morphology of the 10 IPN virus isolates were consistent with those reported for VR-299 isolate by Kelly and Loh (1972) and Cohen et al (1973). Individual capsomeres were difficult to resolve from the electron photomicrographs; however 4

structural components per facet edge were discernible. This appearance suggested that the IPN virus capsid is made up of 92 capsomeres by the formula of Horne and Wildy (1960). Of the smaller particles, 3 components per facet edge were discernible.

These results show that the IPN virion is much larger than the picornavirus and should not be classified as being a member of this group on the basis of size (Melnick, 1971; Andrewes and Periera, 1967).

Figure 28

Electron Photomicrograph of purified VR-299 IPN Virus

Virus was obtained from the second purification in CsCl and negatatively stained with PTA as described in materials and methods.

Magnification: X 89,600

Bar: 100 nm

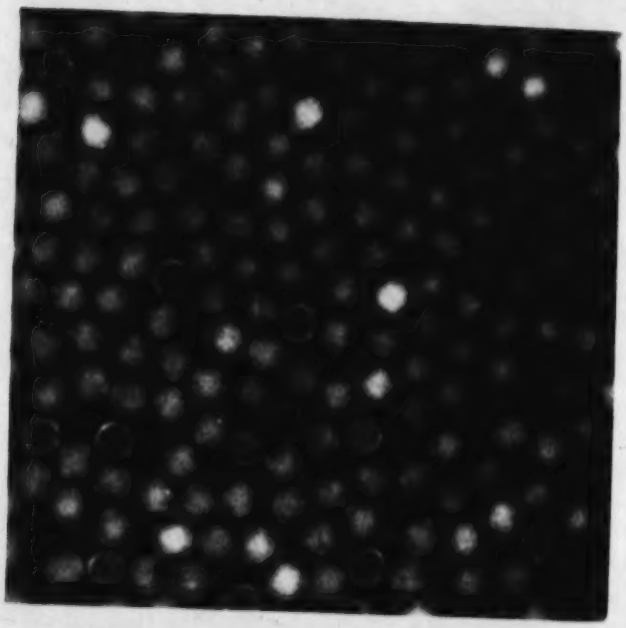
Figure 29

Electron Photomicrograph of purified Jasper IPN Virus

Virus was purified and negatively stained with PTA as described in materials and methods.

Magnification: X 97,000

Bar: 100 nm



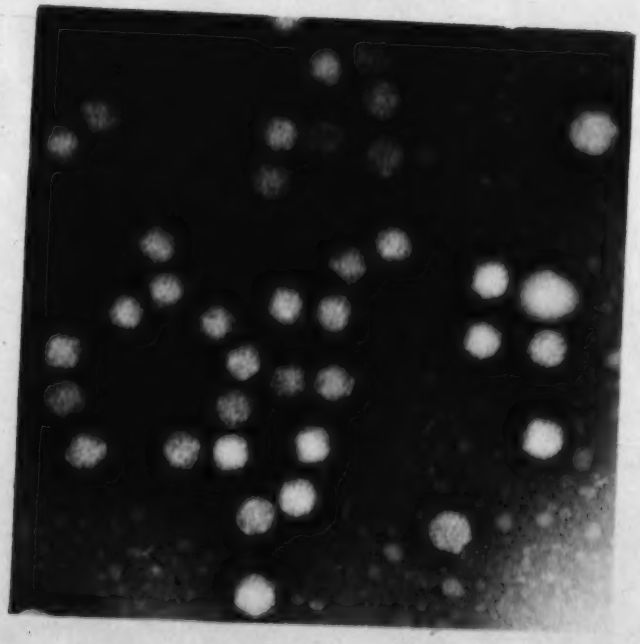


Figure 30

Electron Photomicrograph of purified Fall River (FR) IPN Virus and Latex Particles

Virus was purified and negatively stained with PTA as described in materials and methods. The latex particles measures 88 nm in diameter. FR IPN virus particles measures approximately 74 nm in diameter when compared with the latex particles.

Magnification: X 89,600

Bar: 100 nm

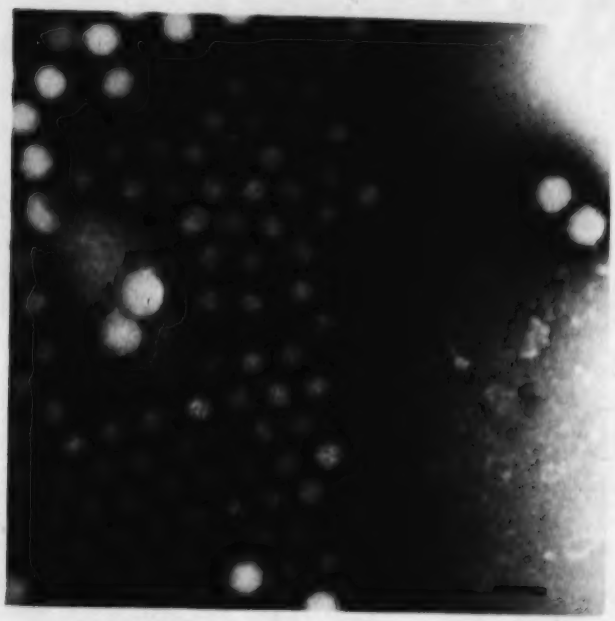
Figure 31

Electron Photomicrograph of purified Buhl IPN Virus

Virus was purified and negatively stained with PTA as described in materials and methods. Virus particles measures approximately 74 nm.

Magnification: X 89,600

Bar: 100 nm



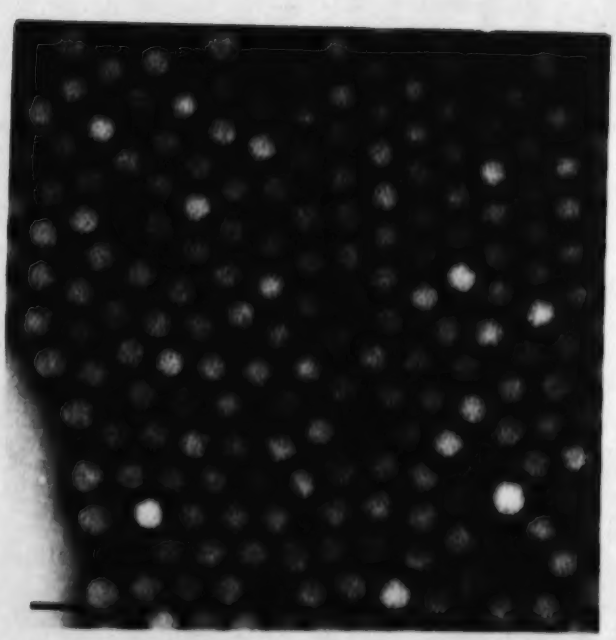


Figure 32

Electron Photomicrograph of purified West Buxton IPN Virus

Virus was purified and negatively stained with PTA as described in materials and methods. A few empty capsids are clearly evident and two smaller particles of approximately 58 nm in diameter are present, and which contained 3 structural components per facet edge.

Magnification: X 89,600

Bar: 100 nm

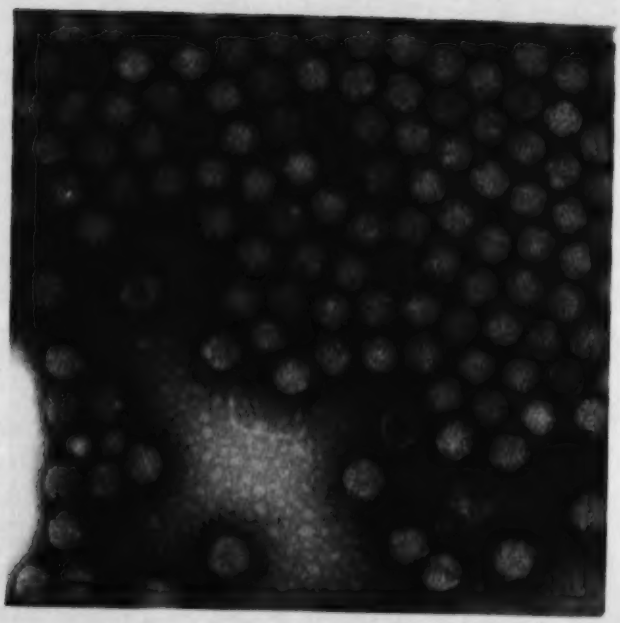
Figure 33

Electron Photomicrograph of purified Reno IPN Virus

Virus was purified and negatively stained with PTA as described in materials and methods. Empty capsids and 58 nm diameter particles are evident.

Magnification: X 89,600

Bar: 100nm



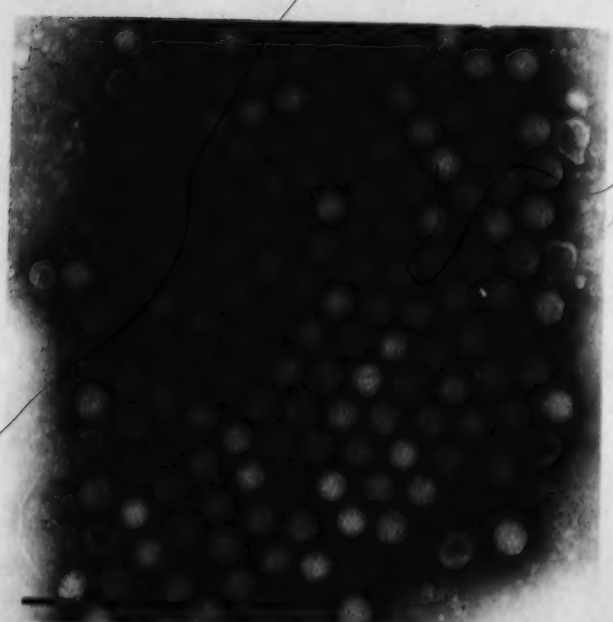


Figure 34

Electron Photomicrograph of purified Powder Mill IPN Virus

Virus was purified and negatively stained with PTA as described in materials and methods.

Magnification: X 89,600

Bar: 100 nm

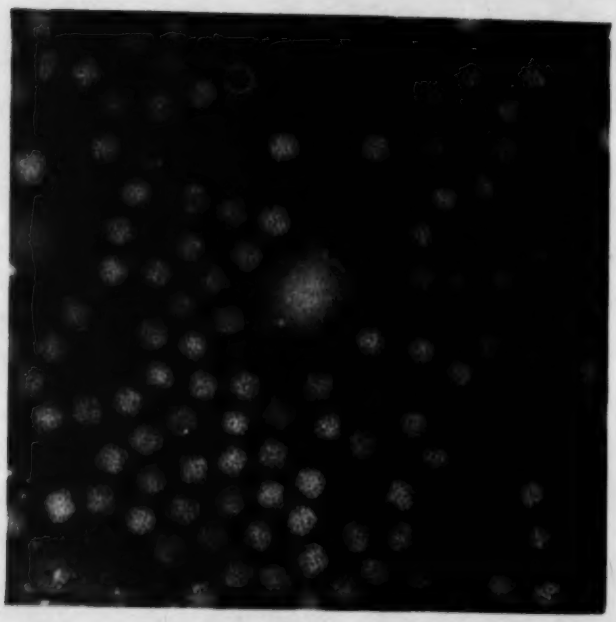
Figure 35

Electron Photomicrograph of purified Western IPN Virus

Virus was purified and negatively stained with PTA as described in materials and methods.

Magnification: X 89,600

Bar: 100 nm



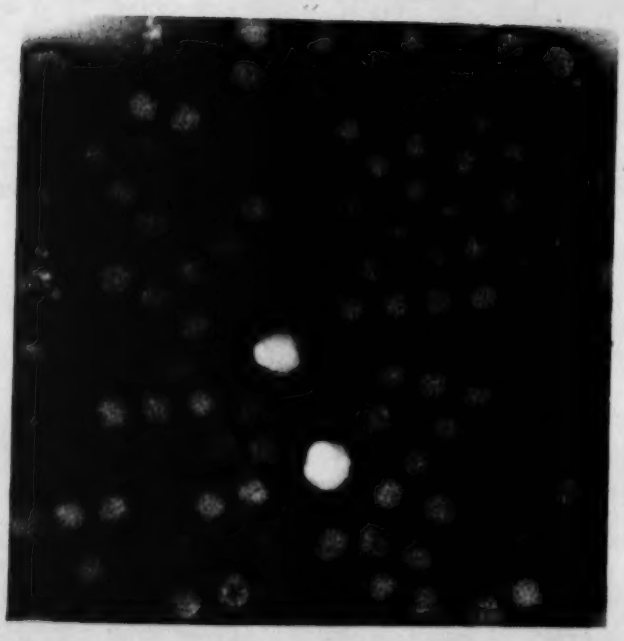


Figure 36

Electron Photomicrograph of purified d'Honnincthun IPN Virus

Virus was purified and negatively stained with PTA as described in materials and methods.

Magnification: X 89,600

Bar: 100 nm

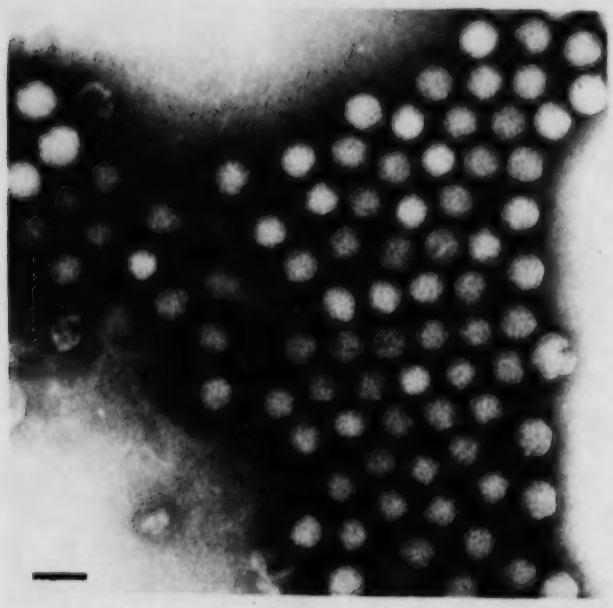
Figure 37

Electron Photomicrograph of purified Bonnamy IPN Virus

Virus was purified and negatively stained with PTA as described in materials and methods. Latex particles of 88 nm diameter are evident.

Magnification: X 89,600

Bar: 100 nm



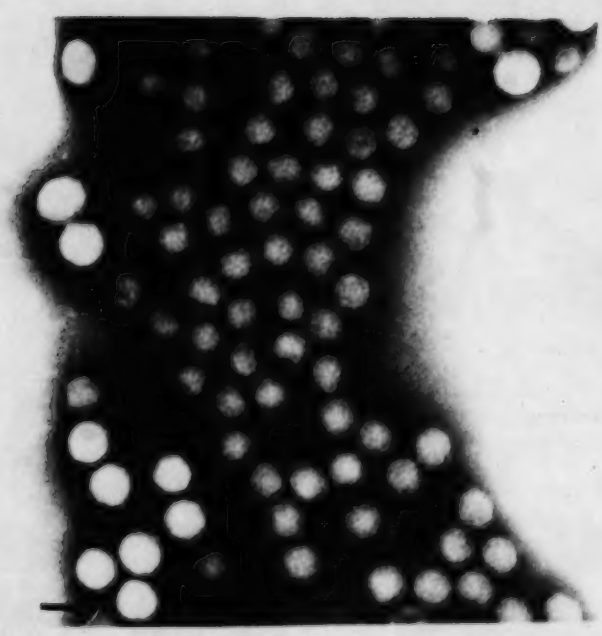


Figure 38

Electron Photomicrograph of Reovirus Type 2 Particles Negatively Stained with PTA

L cells were infected with reovirus type 2 and were harvested and treated with Freon 113. The Freon extracted material containing the virus was layered onto a 20 to 40% CsCl gradient and centrifuged for 16 h at 35,000 rev/min at 4°C in the Beckman L2-65B ultracentrifuge using the SW 50.1 rotor. The virus band was withdrawn and dialyzed overnight against Tris-HCl buffer, pH 7.2 and then prepared for electron microscopic observations as described in materials and methods.

The thick outer shell of the virus capsid is clearly evident in those particles that contained empty cores. The inner protein coat (inner capsid) is discernible in many of the empty particles (arrow). The virion measures 77 nm in diameter.

Magnification: X 103,300

Bar: 100 nm

11. Particle Counts of Virus and Latex Suspensions

The lowered drop method of Pinteric and Taylor (1962) was performed to determine the number of IPN virus particles present in a virus suspension. The procedure is described in materials and methods. Reovirus type 2 was used as a control for the method. In the procedure prescribed by Pinteric and Taylor, the ammonium acetate-ammonium carbonate salt solution contained o.15 M ammonium acetate at pH 7.3; while this was found to work very well with reovirus as shown in Fig 39, IPN virus particles were not observed to be present when shadowed with platinum-palladium unless stained with PTA (Fig 40). This would seem to indicate that the virus particles were embedded within a layer of material on the surface of the titanium grid, and hence were not accessible to platinum-palladium shadowing, in contrast to the latex particle which was clearly shadowed.

Since it became apparent that IPN virus particles could not be resolved by the prescribed methods of Pinteric and Taylor, attempts were made to fix the virus with either formaldehyde or glutaraldehyde. Fixation with 1 % formaldehyde was found to be inadequate while 0.3% glutaraldehyde was found to be sufficient to fix the virus. The concentration of ammonium acetate was found to affect the result. The results shown in Fig 41 and Fig 42 show that ammonium acetate at 0.5 M and 0.15 M concentration failed to allow resolution of the IPN virus particles. What appeared to be virus particles were faintly discernible but not clearly distinct. A concentration of 0.01 M ammonium acetate was found to give the best result. This is shown in Fig 43a and Fig 43b. Distilled water could not be substituted for the ammonium acetate solution.

Figure 39

Platinum-Palladium Shadowing of Reovirus Type 2

A suspension of virus and latex particles (3.5×10^{10} particles/ml) were mixed and layered onto a titanium grid according to the method of Pinteric and Taylor (1962) as described in materials and methods. The latex particles measures 264 nm in diameter.

Magnification: X 53,700

Bar: 200 nm

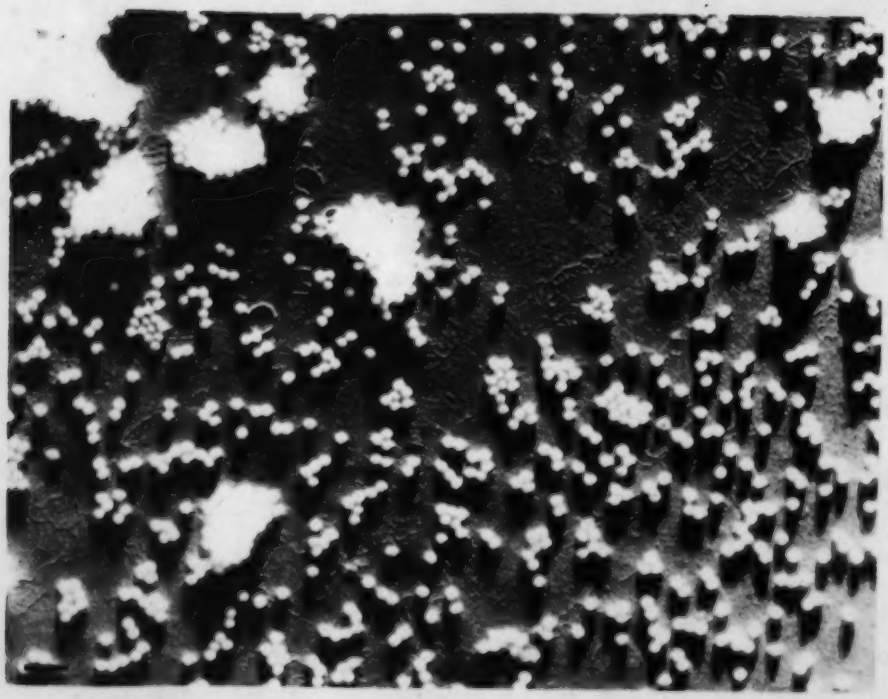
Figure 40

Platinum-Palladium Shadowing of a mixture of VR-299 IPN Virus and Latex particles of 264 nm Diameter

A suspension of virus and latex particles of 264 nm diameter (3.5×10^{9} particles/ml) were mixed and layered onto a titanium grid as described in materials and methods. A small volume of PTA was dispensed onto the shadowed grid. By this means the unshadowed IPN virus particles were made discernible when observed under the electron microscope (arrow).

Magnification: X 53,700

Bar: 200 nm



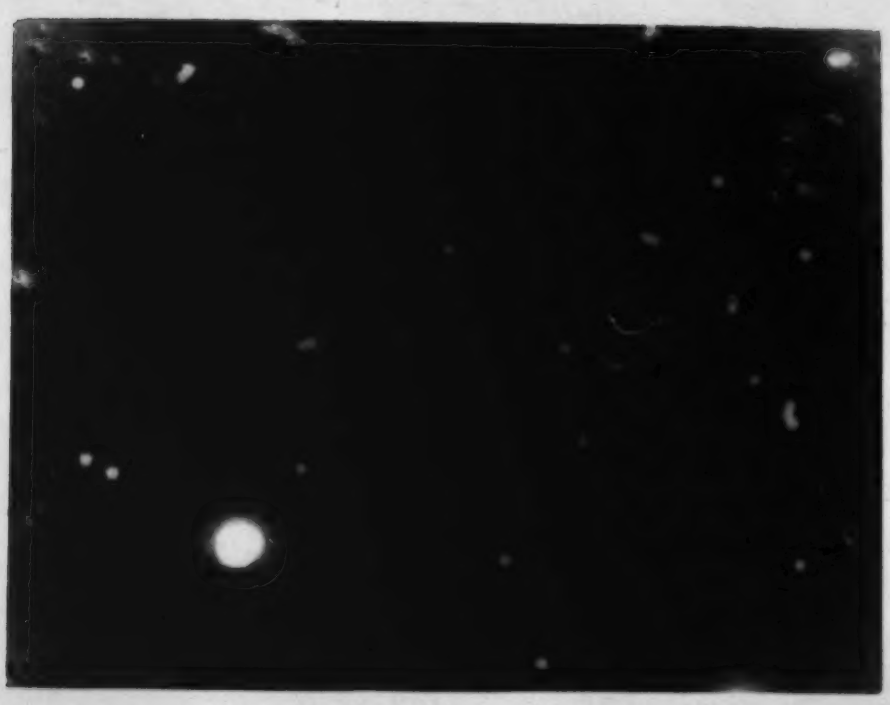


Figure 41

Platinum-Palladium Shadowing of a Mixture containing Latex Particles and VR-299 IPN Virus Particles

Virus and latex particles of 264 nm diameters (3.5×10^{10} particles/ml) were mixed and layered onto a titanium grid according to the method of Pinteric and Taylor (1962) with the exception that 0.5 M ammonium acetate solution was used as the lowering solution.
VR-299 IPN virus particles are not discernible in contrast to the latex particles which are clearly evident (arrow).

Magnification: X 53,700

Bar: 200 nm

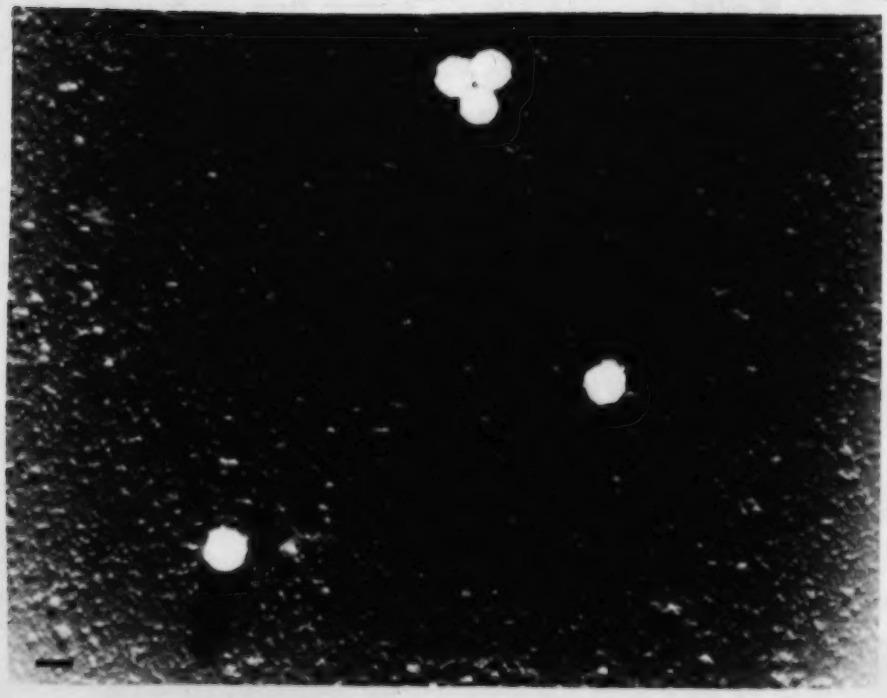
Figure 42

Platinum-Palladium Shadowing of a Mixture containing VR-299 IPN Virus and and Latex Particles

Virus and latex particles of 264 nm diameter were mixed and layered onto a titanium grid according to the method of Pinteric and Taylor (1962). Latex particles are clearly evident while VR-299 IPN virus particles are not.

Magnification: X 53,700

Bar: 200 nm



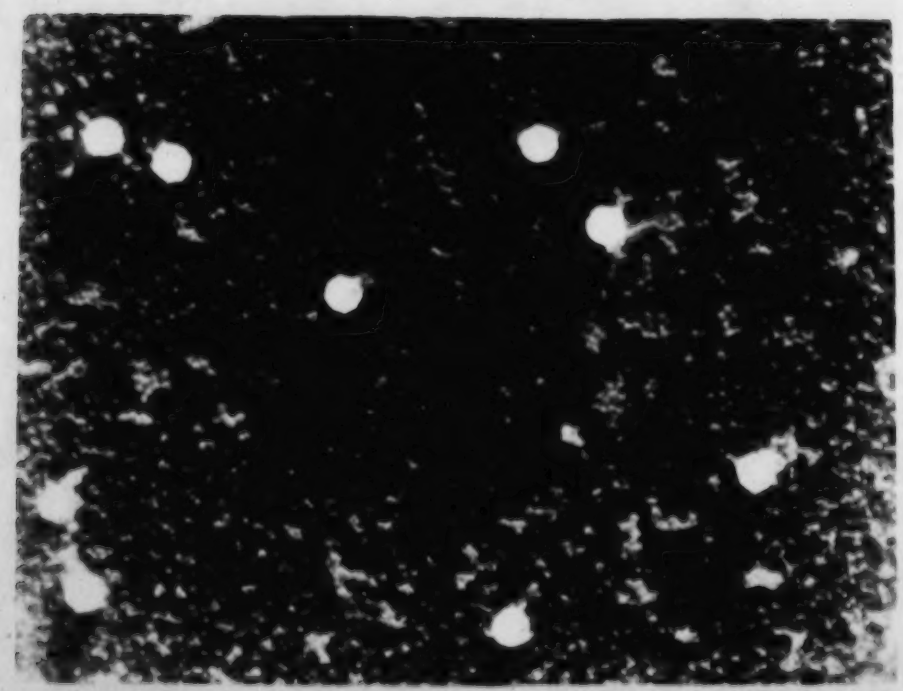


Figure 43a

VR-299 IPN Virus Particle Count

Equal volumes of purified VR-299 IPN virus and 264 nm latex particles (3.5×10^{10} particles/ml) were mixed and prepared for electron microscopy according to the method of Pinteric and Taylor (1962) with the exception that 0.01 M ammonium acetate was used as the lowering solution instead of the prescribed concentration of 0.15 M.

Both latex particles and VR-299 IPN virus particles (arrow) are clearly evident.

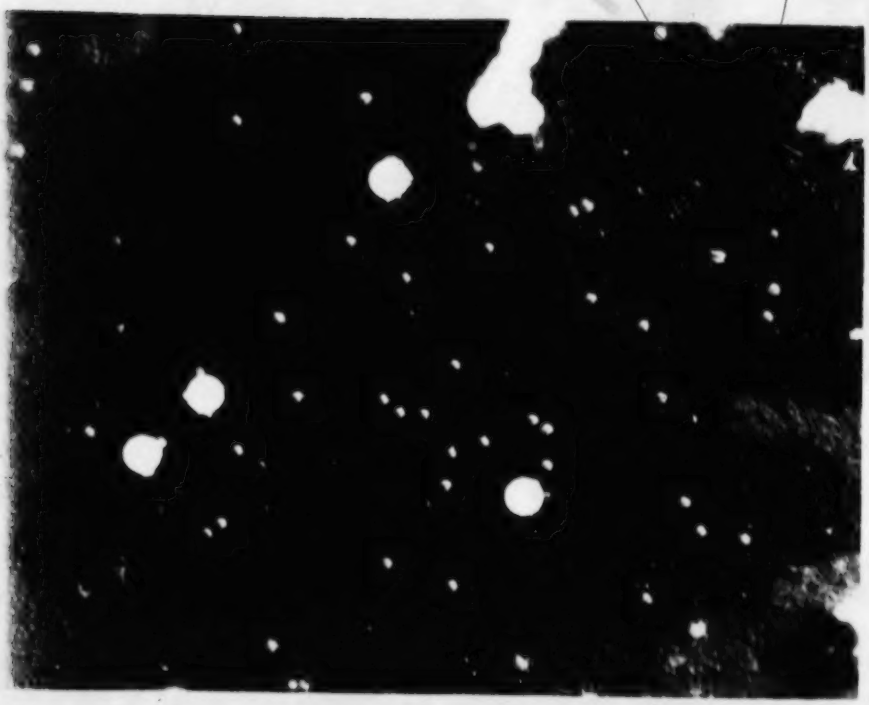
Figure 43b

A Higher Magnification of VR-299 IPN Virus Particles and 264 nm Diameter Latex

A higher magnification of VR-299 IPN virus particles and latex under the same condition as Fig 43a.

Magnification: X 123,200

Bar: 200 nm



Particle numbers were determined as shown in Tables 10, 11 and 12. The average value obtained from 2 grids from two virus samples (Tables 11 and 12) was 178 particles to one PFU. A value of 54 particles to one PFU was obtained with one virus sample (Table 10). Thus, the results show that one PFU is equivalent to about 50 to 200 particles. The ratio may vary from one virus preparation to another.

Table 10

Distribution of 264 nm Latex Particles and VR-299 IPN Virus Particles on a Grid prepared by Lowered Drop Method-Virus Sample 1

Field number	Number of particles in randomly selected fields	
	264 nm latex	IPN virus particles
1	25	271
2	14	118
3	10	63
4	12	168
5	18	173
6	3	144
7	9	300
8	2	125
9	4	160
10	10	301
11	5	450
12	3	101
13	9	541
Total	124	2643

Concentration of latex particles in virus-latex mixture= 3.5×10^{10}/ml

Concentration of virus particles in virus-latex mixture= $\frac{2643}{124} \times 3.5 \times 10^{10}$
$= 7.5 \times 10^{11}$/ml

Concentration of infectious virus= 1.4×10^{10} PFU/ml

PFU:Particle ratio is one PFU:54 particles.

Table 11

Distribution of 264 nm Latex Particles and VR-299 IPN Virus Particles on a Grid prepared by Lowered Drop Method-Virus Sample 2

Field number	Number of particles in randomly selected fields	
	264 nm latex	IPN virus particles
1	23	79
2	21	24
3	42	92
4	12	79
5	10	52
6	16	63
7	17	34
8	30	46
9	44	35
10	27	58
Total	252	562

Concentration of latex particles in virus-latex mixture= 3.5×10^{11}/ml

Concentration of virus particles in virus-latex mixture= $\frac{562}{252} \times 3.5 \times 10^{11}$

$= 7.8 \times 10^{11}$/ml

Concentration of infectious virus = 4.2×10^{9} PFU/ml

PFU:Particle ratio is one PFU:186 particles

Table 12

Distribution of 264 Latex Particles and VR-299 IPN Virus Particles on a Grid prepared by Lowered Drop Method-Virus Sample 3

Field number	Number of particles in randomly selected fields	
	264 nm latex	IPN virus particles
1	20	74
2	20	60
3	30	35
4	19	70
5	22	40
6	41	40
7	27	117
8	55	79
9	13	72
Total	237	587

Concentration of latex particles in virus-latex mixture= 3.5×10^{11}/ml

Concentration of virus particles in virus-latex mixture= $\frac{587}{237} \times 3.5 \times 10^{11}$

$= 8.8 \times 10^{11}$/ml

Concentration of infectious virus= 4.2×10^{9} PFU/ml

PFU:Particle ratio= one PFU:169 particles

12. SDS-Polyacrylamide Gel Electrophoresis of IPN Virus Proteins

SDS-polyacrylamide gel electrophoresis were performed to determine (a) the presence or absence of IPN virus-specific proteins in infected CHSE-214 cells and (b) the polypeptide composition of purified IPN virus isolates.

(a) SDS-polyacrylamide gel electrophoresis of proteins from Freon extracted IPN virus-infected and uninfected CHSE-214 cells

To determine whether IPN virus specific proteins could be detected at various times after infection in CHSE-214 cells by SDS-gel electrophoresis, confluent cultures were inoculated with approximately 1.5 X 10^6 PFU/ml of VR-299 IPN virus per Roux bottle. At 5 and 10 h pi, culture cells were removed from the glass surface by scraping with a rubber policeman. Medium and cells were centrifuged at 6,000 rev/min for 10 min. The supernatant was removed and the cell pellet was resuspended in a small volume of Tris-HCl buffer and treated with an equal volume of Freon as described in materials and methods. Uninfected cells were similarly treated. The Freon extracted proteins were then treated with SDS and 2-mercaptoethanol and electrophoretically separated as described in materials and methods.

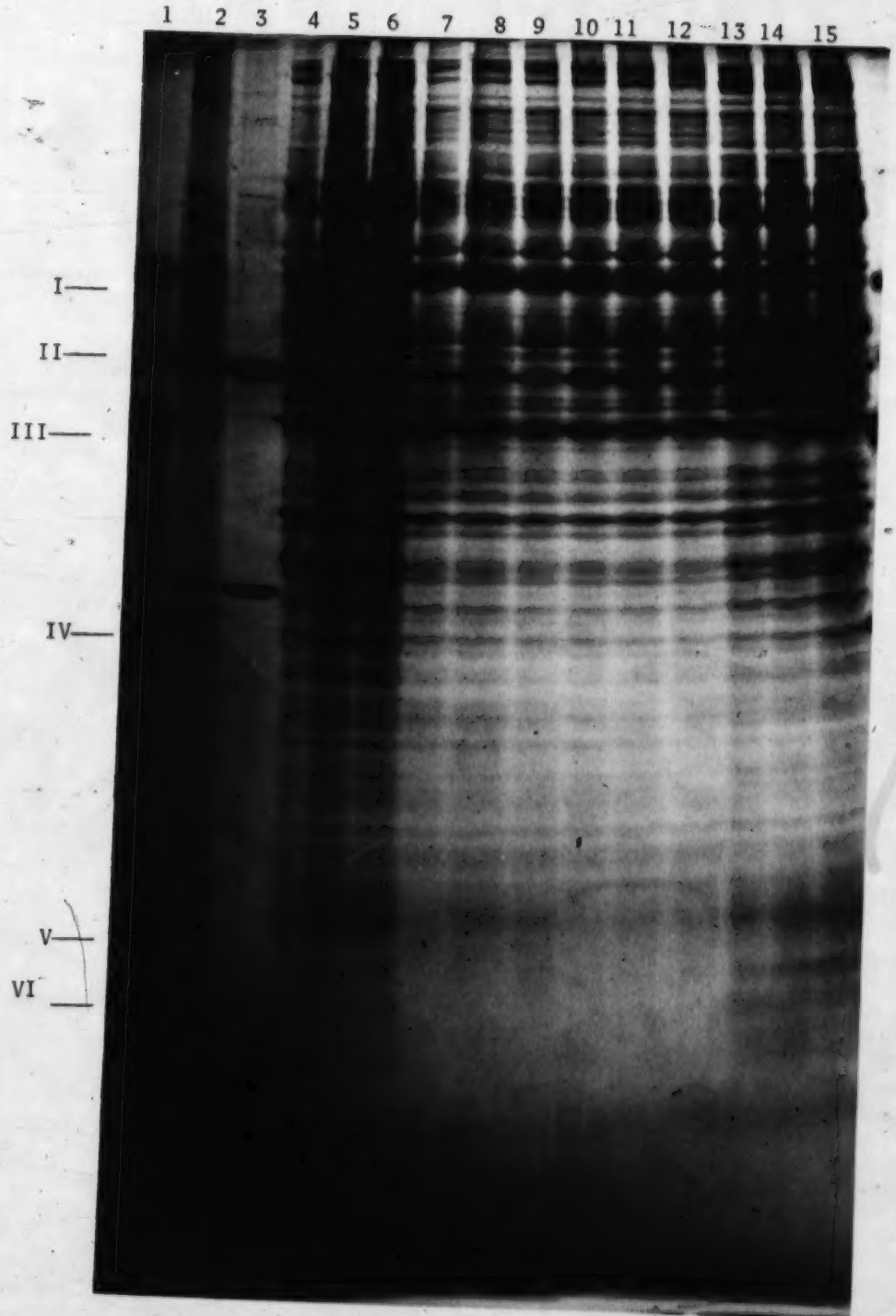
The result in Fig 44 show that it was not possible to resolve IPN virus-specific proteins from cellular proteins which were clearly present in abundance. No significant differences were detected between infected and uninfected cells. Most of the proteins from the Freon extracted virus-infected cells could be separated from the virus band after a single centrifugation in

Figure 44

SDS-Polyacrylamide Gel Electrophoresis of Proteins from Freon extracted IPN Virus-infected and Uninfected CHSE-214 Cells

Slot number	Sample in slot
1	standard protein markers (I to VI)
2	VR-299 IPN virus obtained after one purification in a CsCl gradient centrifugation at 35,000 rev/min for 16 h in a Beckman L2-65B ultracentrifuge using the SW 50.1 rotor.
3	Jasper IPN virus purified twice by CsCl gradient centrifugation at 35,000 rev/min for 16 h.
4, 5, 6	Freon extracted material from uninfected CHSE-214 cells.
7, 8, 9	Freon extract of CHSE-214 cells infected with VR-299 IPN virus at 10 h pi.
10, 11, 12	Freon extract of CHSE-214 cells infected with VR-299 IPN virus at 10 h pi.
13, 14, 15	Freon extracted material from uninfected CHSE-214 cells.

Protein markers		Molecular weight (daltons)
I.	BSA	68,000
II.	γ-globulin	50,000
III.	Ovalbumin	43,000
IV.	Chymotrypsinogen	25,000
V.	Lysozyme	14,300
VI.	Ribonuclease	13,000


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
I
II
III
IV
V
VI

CsCl (slot number 2, Fig 44), but not all. The remaining non-viral proteins were subsequently removed after the second purification in CsCl as described in materials and methods.

Thus, because of the large amounts of cellular proteins present in Freon extracted cells, viral and cellular proteins could not be differentiated in stained gels.

(b) SDS-polyacrylamide gel electrophoresis of purified IPN virus proteins

To determine the polypeptide composition of IPN virus isolates VR-299, Jasper, Bonnamy, d'Honnincthun and FR, the virus were purified twicw in a CsCl gradient for 16 h at 35,000 rev/min, after which time the virus bands were withdrawn, dialyzed and prepared for SDS-gel electrophoresis as described in materials and methods. Protein standards of known molecular weights were also included in the SDS-slab gel (Fig 45 and Fig 46).

The result in Fig 45 show that 3 major protein bands were resolved with molecular weights of 50,000, 30,000, 29,000 daltons designated as VP50, VP30 and VP29 respectively for each of the IPN virus isolate. A minor protein band of about 80,000 daltons or more was detected for VR-299 (slot number 8 and 9, Fig 45). However it was shown to be absent in gels containing virus purified twice in CsCl, the first centrifugation for 16 h and the second for 3 h. The result in Fig 47 show that only 3 protein band were resolved. This would suggest that the high molecular weight may be a cellular contaminant. On the other hand it may be

Figure 45

Separation of IPN Virus Polypeptides by SDS-Polyacrylamide Gel Electrophoresis

IPN virus was purified by two CsCl gradient centrifugations at 35,000 rev/min at 4°C in the Beckman L2-65B ultracentrifuge using the SW 50.1 rotor. The first centrifugation was for 16 h and the second for 6 h.

Slot number	Virus isolate
1	VR-299
2	Jasper
3	Fall River (FR)
4	d'Honnincthun
5	FR
6	d'Honnincthun
7	Bonnamy
8	VR-299
9	VR-299
10	Jasper
11	FR
12	d'Honnincthun
13	Protein standards
14	Protein standards

The protein standards and their molecular weights are: I. BSA(68,000); II. γ-globulin (50,000); III. Ovalbumin (43,000); IV. Chymotrypsinogen (35,000); V. Lysozyme (14,300); VI. Ribonuclease (13,700).

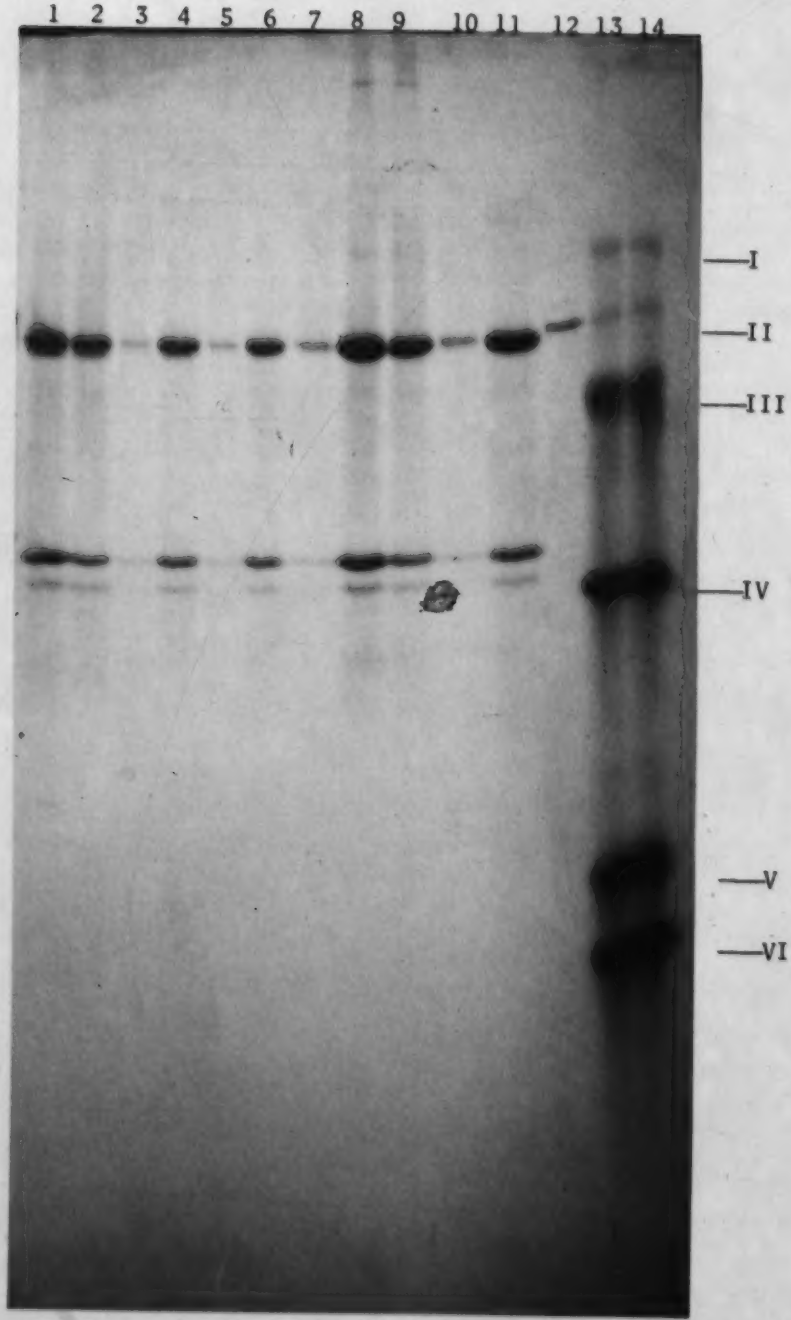

1 2 3 4 5 6 7 8 9 10 11 12 13 14
I
II
III
IV
V
VI

Figure 46

Electrophoretic Mobility of 7 Protein Standards as a Function of their Molecular Weight

The molecular weights are according to Weber and Osborn (1969).

		Molecular weight (daltons)
1.	BSA	68,000
2.	γ-globulin	50,000
3.	Ovalbumin	43,000
4.	Chymotrypsinogen	25,000
5.	Lysozyme	14,000
6.	Ribonuclease	13,700

The positions of the IPN virus polypeptides are indicated by VP50, VP30 and VP29.

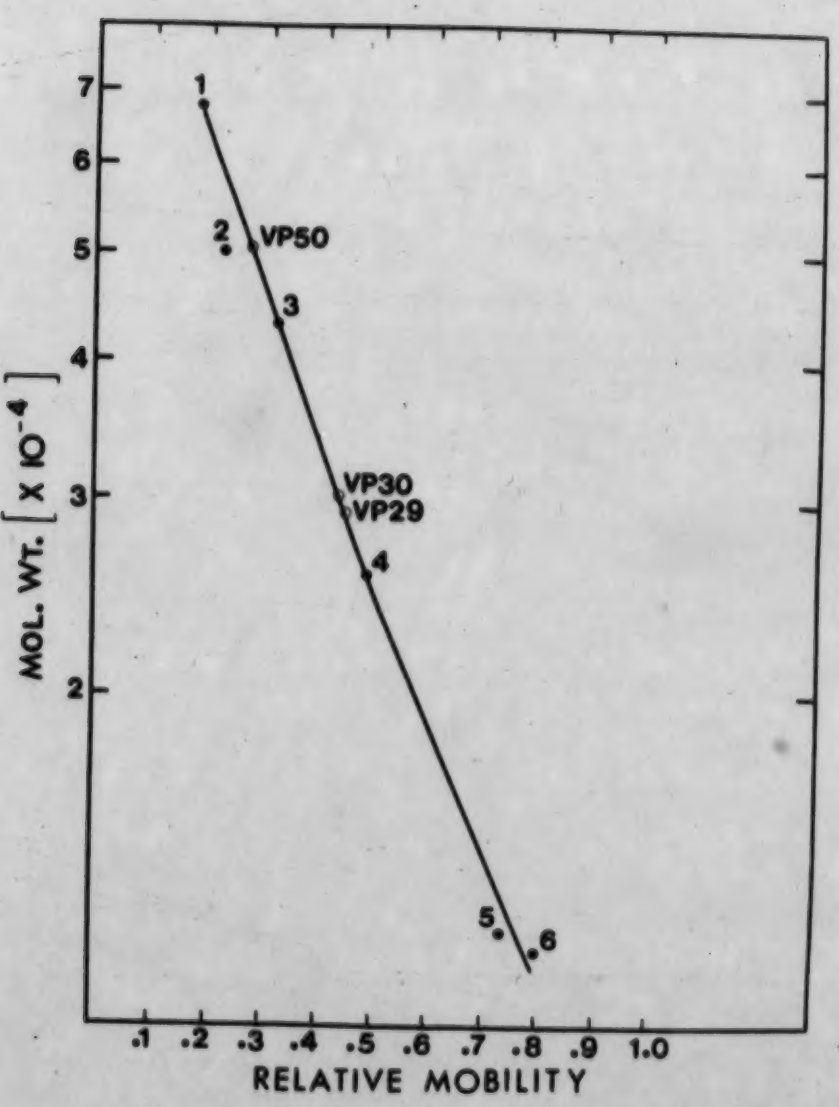

MOL. WT. [X 10⁻⁴]
7
6
5
4
3
2
1
2
VP50
3
VP30
VP29
4
5
6
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
RELATIVE MOBILITY

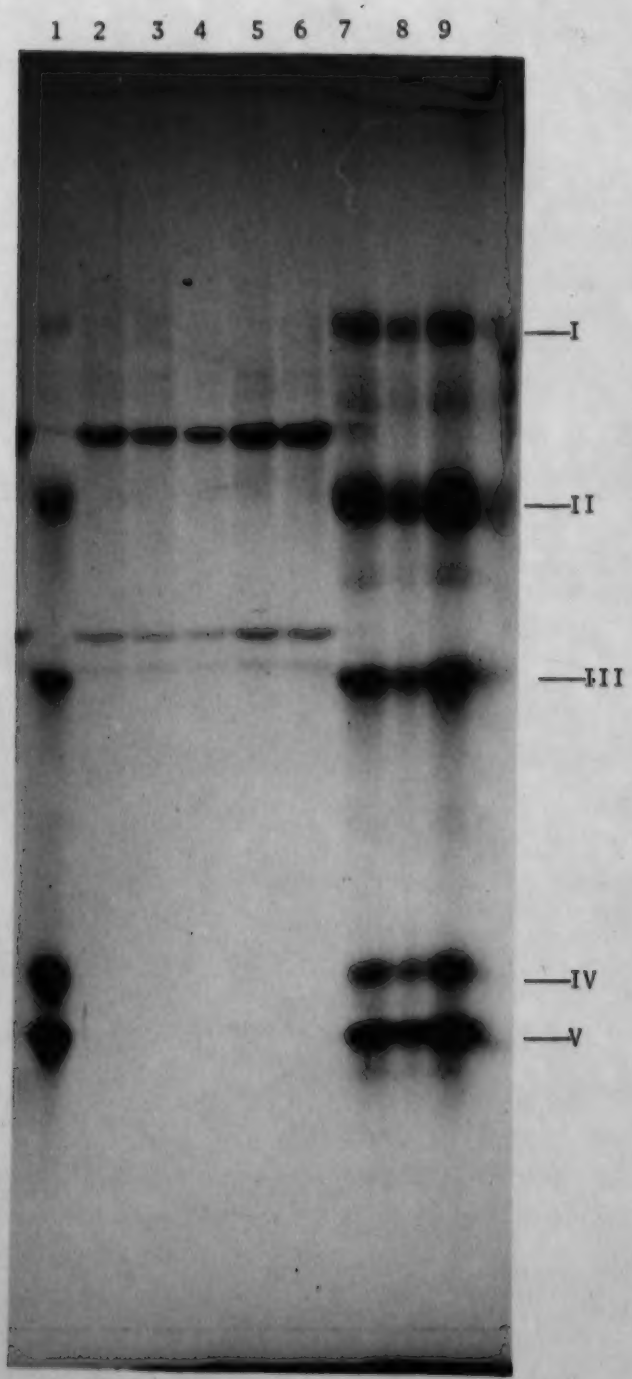
Figure 47

Separation of IPN Virus Polypeptides by SDS-Gel Electrophoresis

IPN virus isolates were purified twice by CsCl gradient centrifugation at 35,000 rev/min for 16 h and 3 h respectively at 4°C as described in materials and methods.

Slot number	Sample
1	Protein standards
2	d'Honnincthun
3	Bonnamy
4	Jasper
5	FR
6	VR-299
7, 8, 9	Protein standards

The protein standards and their molecular weights in brackets are: I. BSA (68,000); II. Ovalbumin (43,000); III. Chymotrypsinogen (25,700); IV. Lysozyme (14,300); V. Ribonuclease (13,700).


1 2 3 4 5 6 7 8 9
I
II
III
IV
V

a component of the virion but is present in very small amounts, consequently it was not detected in stained gels.However overloading of gel for VP50 has failed to detect it.

SDS-gel electrophoresis of purified Reno, Western, West Buxton, Powder Mill and Buhl virus isolates also revealed the presence of only 3 polypeptides of 50,000, 30,000 and 29,000 daltons for each of the virus isolate (Fig 48). The relative amounts of individual protein bands were obtained from densitometer tracings of the polypeptides of each virus isolate (Fig 49). The results in Table 13 show that the VP50 polypeptide consitiuted 60 to 70% of the total virus protein. The amount of VP30 and VP29 was found to vary from isolate to isolate, but the sum of the two constituted approximately 40% of the total protein of each virus isolate. This would seem to indicate that the VP29 polypeptide may be the result of the breakdown of the VP30 polypeptide. Prolonged boiling of purified virus samples in SDS and 2-mercaptoethanol did not alter the pattern of the polypeptide bands in the stained gels (Fig 50). This indicated the possibility that breakdown of VP30 may have occurred prior to denaturation of the virus with SDS and 2-mercaptoethanol.

Figure 48

Separation of IPN Virus Polypeptides by SDS-Gel Electrophoresis

IPN virus isolates were purified as described in materials and methods

Slot number	Virus isolates
1	Protein standards
2	Reno
3	Buhl
4	Powder Mill
5	West Buxton
6	Western
7	Reno
8	Buhl
9	Reno
10	Reno
11	West Buxton

The protein standards are as described in Fig 47

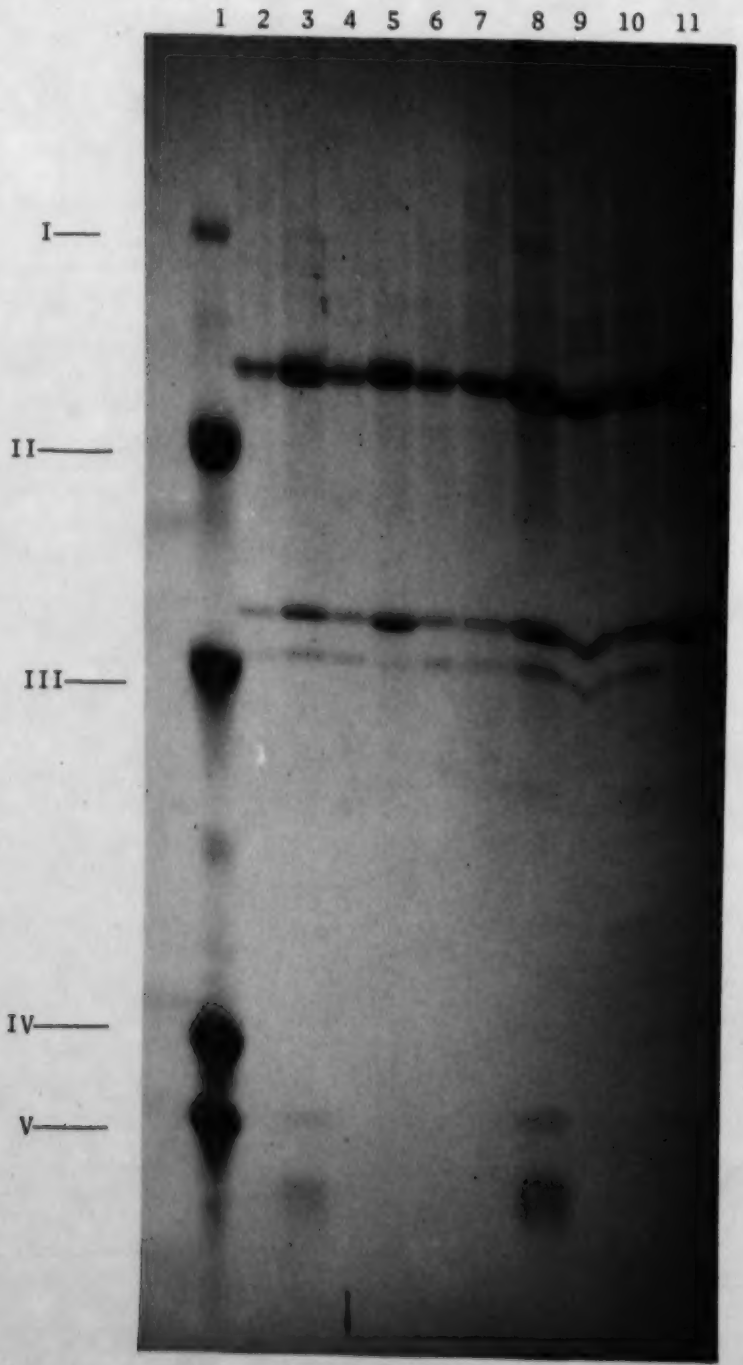

1 2 3 4 5 6 7 8 9 10 11
I
II
III
IV
V

Figure 49

Densitometer Tracings of Virion Polypeptides of 10 IPN Virus Isolates

a. VR-299

b. Jasper

c. FR

d. Bonnamy

e. d'Honnincthun

f. Buhl

g. Reno

h. Western

i. West Buxton

j. Powder Mill

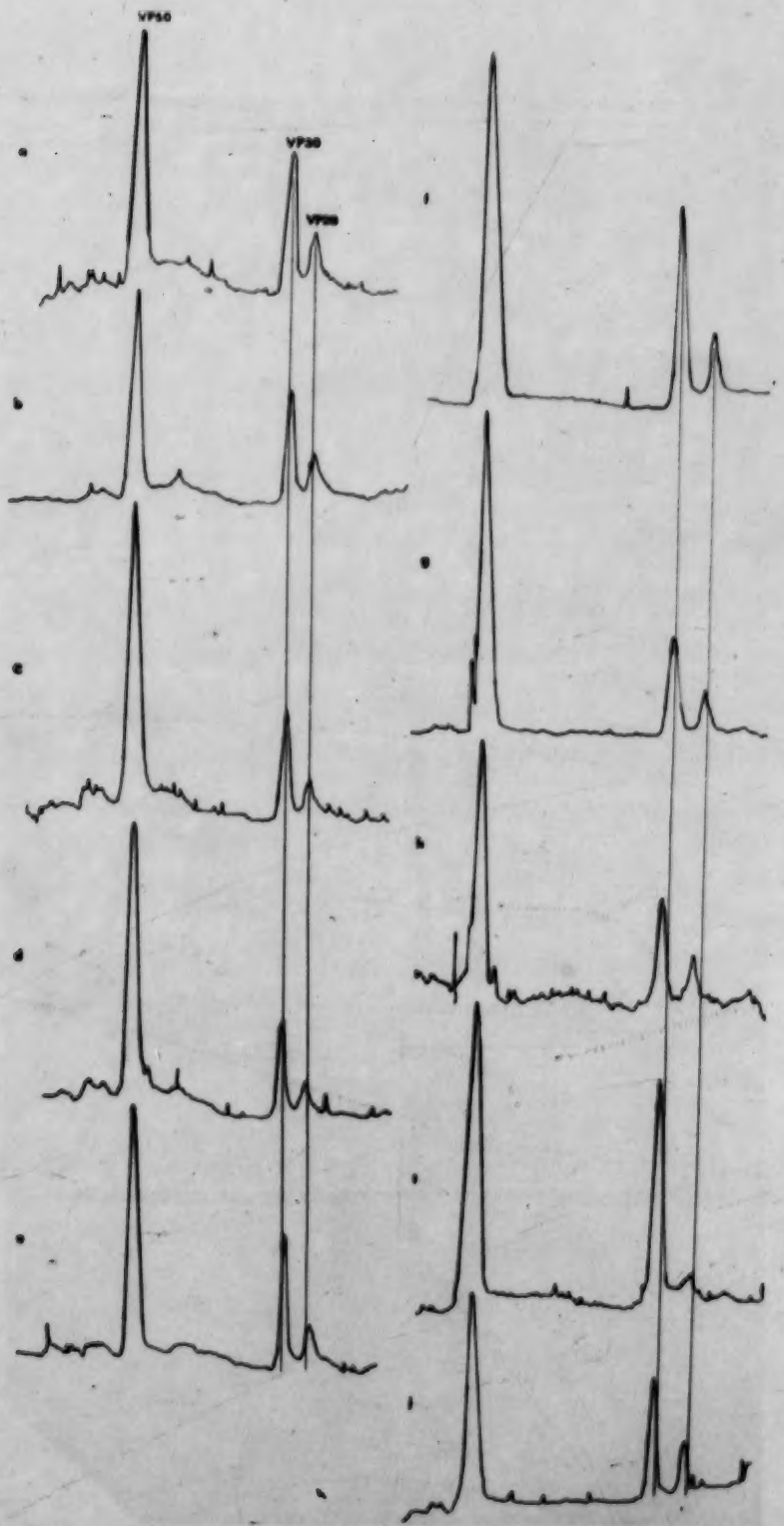

VP10
VP30
VP50

Table 13

Relative Proportions of IPN Virus Polypeptides*

IPN virus isolate	% VP50	% VP30	% VP29	VP30 + VP29
VR-299	64	27	9	36
Jasper	70	20	10	30
Fall River	63	25	12	37
Bonnamy	61	22	17	39
d'Honnincthun	61	28	11	39
Reno	60	30	10	40
Western	60	30	10	40
West Buxton	60	30	10	40
Buhl	60	25	15	40
Powder Mill	60	30	10	40

* The relative proportions of each polypeptide were determined from densitometer tracings of stained gels as shown in Fig 49. Each polypeptide peak was cut out, weighed and expressed as a percentage of the total weights of all three peaks (taken as 100% of viral protein).

Figure 50

Effect of Boiling on the Distribution of Virion Polypeptides in SDS-Polyacrylamide Gel Electrophoresis

IPN virus isolates were purified as described in materials and methods.

Slot number	Treatment
1-5	unboiled
6-10	boiled for 1 min
11-15	boiled for 2 min
16-20	boiled for 4 min

	Virus isolates
1, 6, 11, 16	VR-299
2, 7, 12, 17	FR
3, 8, 13, 18	Jasper
4, 9, 14, 19	Bonnamy
5, 10, 15, 20	d'Honnincthun

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

DISCUSSION

Several observations in this study show that CHSE-214 cells can be routinely employed for the propagation of IPN virus, for the titration of virus infectivity by the plaque assay method and for the diagnosis for IPN virus infection. In IPN virus-infected CHSE-214 cells, the cpe that resulted was found to be characteristically different from the cpe observed in virus-infected RTG-2 cells. In addition, plaque morphology in both cell lines was also distinctly different. Such gross morphological differences in cpe and plaque formation are useful for the study of IPN virus infection and perhaps also for the diagnosis of IPN virus itself. In RTG-2 cells, the resultant plaque morphology is characteristic of a particular virus infection (Wolf and Quimby, 1971). However, no studies were carried out to determine whether plaque morphology in CHSE-214 cells is characteristic of IPN virus infection or of the cell itself. McCain et al (1971) employed CHSE-214 cells for plaque neutralization of three salmonid viruses, the sockeye salmon virus, the Sacramento River chinook virus and infectious hemapoietic necrosis virus, but no information was given regarding the plaque morphology or cpe in cells infected with these viruses. Despite the lack of information, the very fact that distinctive plaque morphology and cpe are observed in virus-infected CHSE-214 cells would appear to warrant the use of this cell line for the study of IPN virus.

The observation that the number of plaques that were formed in CHSE-214 cells was linearly related to the concentration of virus

inoculated showed that this cell line can be employed for titrating virus infectivity. The observation that there was no significant difference in the number of plaques formed in either CHSE-214 or RTG-2 cells indicated that CHSE-214 cells have the same efficiency as RTG-2 cells in the development of plaques.

The appearance of cpe in infected CHSE-214 cells was indicative of the rate of virus multiplication in the cell. Maximum virus production was achieved when cpe was at its most advanced stage (4+). At low multiplicity of infection (0.01 PFU per cell), maximum cpe and virus production was attained within 3 days after infection. An average of 2.5×10^8 PFU/ml of culture media was normally obtained from Roux bottles of infected CHSE-214 cells. An important observation in this study was that CHSE-214 cells could be maintained in Roux bottles for as long as 6 to 8 weeks without medium change. This was longer than the time normally required for the maintenance of RTG-2 cells, which was about 4 weeks.

The above observations thus show that CHSE-214 cells can be employed as readily as RTG-2 cells for the study and detection of IPN virus.

When the stability of VR-299 IPN virus under various pH conditions and low temperature was studied, it was found to be stable at pH 3 to 10 for 1 h at room temperature and at 4°C for 2 months. At much lower temperatures of -65°C, more than 99% of the infectivity was lost after storage for 5 months. McMichael et al (1975) reported that 40% of the infectivity of VR-299 IPN virus was lost after storage for 2 months at -60°C. However, no attempts were made to determine the

rate of inactivation at this low temperature. In this study, the Jasper isolates was found to be more readily inactivated than VR-299 IPN virus at 4°C, -20°C but not at -65°C. Other isolates have been reported to be less stable than the VR-299 isolate. McMichael et al (1975) reported that of the three isolates VR-299, CTT and COHO, VR-299 was the most stable at -60°C; while a loss of more than 70% of the infectivity of the other isolates occurred after storage for 2 months compared to a 40% loss of infectivity for the VR-299 IPN virus isolate. Survival data for other IPN virus isolates are few and meaningful comparisons cannot therefore be made at this time.

The observation that IPN virus was unstable over a long period of time even at very low temperature suggests that non-infectious virus particles were likely to be present in virus samples that have been subjected to freezing and thawing. Thus, those virus isolates that are more readily inactivated by low temperature and changes in pH conditions would be expected to have a greater proportion of non-infectious virus particles. Serological studies using reciprocal cross-neutralization tests by McMichael et al (1975) have indicated such a possibility. The presence of defective particles may affect the titre of the neutralization tests when attempts are made to serotype the various isolates of IPN virus. More careful studies on the stability and serology of all the IPN virus isolates would be required to properly evaluate their relationship.

When the multiplication cycle of VR-299 and Jasper IPN virus in CHSE-214 cells at 18°C was followed, the growth curves of the two virus isolates were found to be essentially the same in CHSE-214 cells as in other fish cell systems such as RTG-2 (Malsberger and Cerini, 1965),

BF-2 (Argot, 1965), GF (Moewus-Kobb, 1965), FHM (Bonnadiere et al, 1976) and SWT cells (Kelly and Loh, 1975). A latent period of about 2 h was detected for both VR-299 and Jasper IPN virus in CHSE-214 cells. Maximum virus production was attained at 10 h pi for the VR-299 isolate and 24 h pi for the Jasper isolate. Latent periods of 3 to 5 h were detected in other fish cell systems for VR-299, with maximum virus production from 15 to 24 h pi. Virus multiplication was reported to be affected by temperature, for example, it was observed that VR-299 IPN virus did not multiply at 33°C in FHM cells (Gravell and Malsberger, 1965), nor at 30 °C in SWT cells (Kelly, 1972), but the virus was capable of multiplying at 16°C in SWT cells, a temperature normally inhibitory to cell growth (Kelly, 1972). Growth of IPN virus at 16°C in CHSE-214 cells was not determined, although this is close to the 18°C that was employed throughout this study. VR-299 IPN virus readily formed plaques at 20°C in RTG-2 cells (Wolf and Quimby, 1973) and has been observed to cause cpe in CHSE-214 cells at 23°C.

The effect of IPN virus infection on the synthesis of DNA and RNA in infected CHSE-214 cells has not been studied. In virus-infected SWT cells, the synthesis of total RNA is enhanced; continuing throughout the virus multiplication cycle. Maximum levels of synthesis was found to parallel the exponential production of infectious virus (Kelly and Loh, 1975). The synthesis of DNA was found to be depressed in infected SWT cells (Kelly and Loh, 1975). These results are basically similar to other observations made for IPN virus in othe cell cultures such as BF-2 (Argot, 1969) and RTG-2 cells (Nicholson, 1971). The general agreement among workers using various fish cell systems would seem to suggest that

very little differences would be expected regarding the effect of IPN virus infection on RNA and DNA synthesis in infected CHSE-214 cells.

Evidence presented in this study showed that IPN virus can be concentrated and purified by the combined techniques of Freon extraction of infected cells and PEG precipitation of culture medium containing virus followed by two cycles of purification in CsCl gradients with very little loss of infectivity. The results are in basic agreement to that reported by Cohen et al (1973), although their quantitative data were rather limited. The use of Freon and PEG would seem to be the method of choice for the concentration and purification of IPN virus when compared to other methods such as pelleting the virus by several high speed centrifugations (Kelly and Loh, 1972). As shown in this study, a significant loss of infectivity resulted when the latter method was employed (Table 2), only 2.2% of the original infectivity was recovered after one centrifugation in a CsCl gradient.

Few data are available regarding the recovery of infectious virus during purification procedures. Thus, the results obtained in this study cannot be critically compared to results obtained in other studies. The apparent lack of data is surprising, if not startling, when one considers the voluminous reports that are available in the literature on IPN virus. The results in this study showed that the concentration and purification of IPN virus has been achieved with satisfactory yields.

In infectivity assays using the plaque assay method, plaque forming units do not represent the number of virus particles that are actually present in a virus sample. The results obtained in this study show that one PFU is equivalent to 50 to 200 particles. The ratio may be

greater or smaller depending on the number of virus samples, and the conditions under which plaquing was performed, and therefore may vary from one preparation to another. This has been shown by the different values obtained for Reovirus type 1. Under optimal conditions for plaquing, the ratio of PFU to particles has been reported to be as low as 1:2 (Wallis et al, 1964), while the ratio of chymotrypsin-activated reovirus particles to infectious units as measured by fluorescent cell count was as low as 1:1 (Spendlove et al, 1970). Yet these ratios are 25 to 200 times lower than those commonly observed in routine assays of purified virus stocks (Joklik, 1974).

The presence of large numbers of non-infectious IPN virus particles could account for the interference with infection that has been consistently observed throughout this study in virus-infected CHSE-214 cells. It was found that high concentrations of undiluted stock virus that had been serially passaged many times in CHSE-214 cells failed to produce cpe when inoculated into healthy CHSE-214 cells unless the virus inoculum was diluted 100 to 1000 fold. Further studies are required to show that the interference was due to defective interfering particles and not to some other cause such as the presence of interferon. Auto-interference has previously been reported for IPN virus-infected RTG-2 and AS cells (Malsberger and Cerini, 1963; Nicholson and Dunn, 1974), but the cause of the interference has not been determined.

An examination of the electron photomicrographs of the 10 IPN virus isolates reveals that they all have the same morphological features. They were non-enveloped, icosahedral in structure with an average diameter of 74 nm and lack the inner capsid that is characteristic of

reovirus. The icosahedral nature of purified virions have been previously reported for VR-299 IPN virus by Kelly and Loh (1972) and Cohen et al (1973) and confirms the earlier observations of Moss and Gravell (1969). In thin sections of infected cells, the particle size was reported to be 55 to 65 nm (Wolf and Quimby, 1971; Moss and Gravell, 1968, 1969; Ball et al, 1971). The reason for the difference in sizes is unknown. It may be due to the fixation procedures that were employed. On the other hand, it is also possible that the slightly larger size of 74 nm reported for purified virions may have been due to procedures employed during virus purification and concentration, such as sedimenting in CsCl gradients at high speed. Partially degraded particles from such preparations appeared expanded and larger than intact virions in negatively stained preparations (Cohen et al, 1973). This could be the result of particle disruption. In this study, the particle sizes were determined relative to that of latex particles of 88 nm diameter. Although the size distribution of the particles were fairly constant with a standard deviation of $\pm$ 5%, it is not known whether the latex particles have expanded or contracted in size under the conditions used, although a comparison of the latex size with that derived from latex and catalase crystals revealed identical values. A recent report by Dobos et al (1977) showed that the particle diameter of VR-299 IPN virus in the hydrated state was 64 nm while the dehydrated state was 59.3 nm. The sizes were determined relative to that of beef catalase crystals. In this study, the individual capsomeres were difficult to resolve in electron photomicrographs of negatively stained preparations of IPN virus isolates, although on closer examination, 4 structural units could be distinguished per viral facet edge.

Using the formula of Horne and Wildy (1962), 92 capsomeres were calculated to be present on the virion surface. Moss and Gravell (1962) and Kelly (1972) predicted the same value on the basis of similar observations. However, unless more details of the surface structure can be resolved, the actual number of capsomeres cannot be established with certainty.

The observations that the buoyant densities of the IPN virus isolates were all identical at 1.33 g/cc in CsCl corresponds with the results obtained from electron microscopy and SDS-gel electrophoresis of purified virions.

In SDS gels, three protein components of 50,000, 30,000, 29,000 daltons designated as VP50, VP30 and VP29 respectively were resolved for all the IPN virus isolates. The relative proportions of each polypeptide were found to be fairly constant. Only the relative proportion of VP30 and VP29 was found to vary from isolate to isolate but the sum of the two constituted about 40% of the total protein. It is probable that VP29 is a degradation product of VP30 since tryptic digests of VP30 and VP29 were identical (P. Dobos, personal communication). In contrast to previous reports, there was no evidence of the 80,000-125,000 dalton protein reported to be present in isotopically labelled virions (Loh et al, 1974; Cohen et al, 1973; Dobos, 1977; Dobos et al, 1977). Although this protein may be present in too small amounts to be visible in stained gels it should be noted that polypeptides of this size did not become visible even when gels were overloaded with VP50. The 100,000 and 90,000 dalton proteins reported by Dobos (1977) constitute about 10% of the total protein, and could only be detected in UV-irradiated virus-infected RTG-2

cells, but not in UV-irradiated uninfected cells. On the other hand, the high molecular weight protein was reported to constitute only 2.5 to 3% by Cohen et al (1973) and Loh et al (1974). The presence of the 100,000 dalton protein could not be explained by aggregation of two 50,000 dalton polypeptides, nor could the 80,000 dalton protein be due to aggregation of the 50,000 and 30,000 dalton polypeptides since SDS-protein complexes migrate as extended polypeptides. If aggregation of the two 50,000 dalton chains occurred, the migration would have to indicate a polypeptide of molecular weight less than 100,000 daltons. Dobos et al (1977) has more recently reported the presence of only one protein of 90,000 daltons and which he suggested may be associated with the RNA genome. Such a polypeptide if present once per virion would represent about 0.2% of the total virion protein. The author has however calculated that there were 22 molecules of this polypeptide within the IPN virion and would therefore constitute about 4% of the total virion with a molecular weight of 55×10^6 daltons. To date, the nature and function of this protein is unknown. The presence of 7 polypeptides in the report by Loh et al (1974) is not substantiated by Cohen et al (1973) nor by this study. Finally the available data indicates that the high molecular weight polypeptide is probably not a cellular contaminant, although it is present in inconsistent amounts and was not consistently detected in SDS-gels (Cohen et al. 1973).

On the basis of the evidence presented in this study, IPN virus is not related to the reoviruses. Although it is similar in size and morphology to the mammalian reoviruses, the present study indicates that IPN does not contain a readily discernible inner capsid has has been described

for the reoviruses (Vasquez and Tournier, 1962; Loh et al, 1965; Mayor et al, 1965). To further strengthen the argument, reovirus type 2 has a buoyant density of 1.37-1.38 g/cc (Gomatas and Tamm, 1963; Loh and Oie, 1969) in contrast to the value of 1.33 g/cc reported for IPN virus by Kelly and Loh (1972), by Dobos (1976) and in this study. The RNA genome of reovirus is similar to the RNA genome of IPN virus with respect to its double-stranded nature. The former yields 10 discrete and unique RNA species in polyacrylamide gels regardless of the means used to liberate the genome. These 10 species are present in equimolar amounts and possess an aggregate molecular weight of 15 X 10^6 daltons (Shatkin et al, 1968; Vasquez and Kleinschmidt, 1968; Watanabe et al, 1968; Granboulin and Niveleau, 1967) and additional single stranded RNA molecules that include PolyA (Bellamy et al, 1972; Nichols et al, 1972; Stoltzfus and Banerjee, 1972). Other viruses which yield multiple genomic segments similar to reovirus are wound tumor virus (Kalmakoff et al, 1969), rice dwarf virus (Fujii-kawata et al, 1970), cytoplasmic polyhedrosis virus (Lewandowski and Millward, 1971) and members of the Orthoreovirus and Orbivirus (Joklik, 1974). The RNA of IPN virus however yields only 2 discrete RNA species of 2.3 and 2.5 X 10^6 daltons (Macdonald and Yamamoto, 1977; Dobos, 1976) with no evidence as yet of PolyA components or other components in polyacrylamide gels. Reoviruses are dissociated into their component polypeptides on treatment with SDS and 2-mercaptoethanol. Electrophoresis in polyacrylamide gels of the resulting mixtures of SDS-polypeptide complexes reveals the presence of seven polypeptides ranging from 34,000 to 155,000 daltons, while only three major polypeptides were resolved in SDS gels in this study, two major and one minor

polypeptides were resolved by Cohen et al (1973) and four polypeptides by Dobos et al (1977) for IPN virus. The virions of all 3 serotypes of reovirus agglutinate human erythrocytes of all four ABO blood groups (Brubaker et al, 1964). IPN virus on the other hand does not have either hemagglutination nor hemadsorption properties (Malsberger and Cerini, 1963). Thus, IPN virus cannot be grouped within the family Reoviridae at the present time, instead it should be included within the Diplornavirus group, but distinct from the Reoviridae. Such a group may include viruses that have been isolated in different hosts, for example the infectious bursal disease (IBDV) from chickens (Nick et al, 1976).

In conclusion, there were no differences in the morphology, buoyant density, or polypeptide composition among the 10 isolates of IPN virus. The small compositional differences of VP30 and VP29 discussed above is most likely due to variables other than strain differences. The fact that the causative agent of IPN disease is morphologically and physiochemically identical when isolated from different regions around the world is consistent with the idea that the viral agents comprise a single unique species and possibly originated in the eastern United States (Wolf, 1972). Alternatively, the agents could be a family of closely related viruses as suggested by some of the serological evidence (Wolf, 1972). A final answer will come from either peptide mapping of the polypeptides, oligonucleotide mapping of the RNA species, or careful cross-neutralization studies on purified virus with controlled input of antigens.

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